DISCOVERY, SURVEILLANCE, AND MANAGEMENT OF HERBICIDE-RESISTANT AMARANTHUS SPP

BY

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THESIS

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ABSTRACT

Amaranthus species are considered key driver weed species within Midwestern agricultural production systems. Herbicides are often relied upon for the control of *Amaranthus* spp. In recent years, herbicide resistance at multiple sites of action, such as 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and protoporphyrinogen oxidase (PPO), have become widespread within a narrow subset of the *Amaranthus* genus, such as *A. tuberculatus* and *A. palmeri*. Species within the genus possesses a distinct geographic distribution. Maintenance of these geographical boundaries can prevent the spread of more competitive species into new regions. The concept of geographical boundaries can be further applied to herbicide-resistant and susceptible biotypes of a given species. By maintaining isolation of herbicide-resistant populations from herbicide-sensitive populations, the spread of resistance may be mitigated. The objectives of this Master's research were the i) identification of single nucleotide polymorphisms (SNPs) which delimit common weedy and cultivated *Amaranthus* spp., ii) development of quantitative assays to detect key *Amaranthus* spp., *A. tuberculatus* and *A. palmeri*, in mixed seed samples, iii) investigation of herbicide resistance in soybean fields of Ohio, iv) characterization of herbicide resistance within *A. retroflexus* accessions from Ohio, v) quantification of gene flow between *A, tuberculatus* and *A. albus,* and vi) development of a full plant regeneration procedure for *A. tuberculatus* through tissue culture. The research objectives were addressed utilizing a wide range of resources, including in-house and National Plant Germplasm System (NPGS) germplasm collections, population collections from Ohio and interspecific crosses.

Amaranthus spp. delimiting barcodes in the nuclear ribosomal *internal transcribed spacer* (ITS) region were identified utilizing 75 sequences from the National Center for Biotechnology Information (NCBI) and 92 accessions from in-house collections and the NPGS. Nine species-specific barcodes were identified, and five species were delimited with single SNPs within the *ITS* region. SNPs specific to key weedy species *A. tuberculatus* and *A. palmeri* were utilized to develop quantitative polymerase chain reaction (qPCR) assays for species detection in mixed seed samples. A DNA extraction procedure from 100 seed samples was optimized to complement the qPCR assays, allowing high-throughput screening of seedlot scale samples.

Amaranthus tuberculatus populations were collected in the 2016 growing season in Ohio as part of a long-term herbicide resistance surveillance initiative. A total of 24 populations were investigated for herbicide resistance to the photosystem II inhibitor atrazine, PPO-inhibitor lactofen, and EPSPS-inhibitor glyphosate. Resistance to atrazine and lactofen were sporadically observed, while high levels of resistance to EPSPS inhibitor glyphosate were consistently observed throughout the state. Continued surveillance of herbicide-resistant population is critical for the maintenance of sensitive populations to common herbicides.

Accessions of *A. retroflexus* from Ohio were identified as potentially resistant to PPO-inhibitor fomesafen and glyphosate in greenhouse screening. Herbicide resistance was characterized through a ten-step dose response to both herbicides. Four-parameter log-logistic models were constructed for resistant and sensitive accessions to each herbicide. A significant difference between the accession in the dose required for 50% inhibition (ED50 value) and lower limit were observed for response to fomesafen (P-value<0.01). No significantly different parameter relationships were observed for response to glyphosate. Sequence comparison of the PPO gene between resistant and sensitive accessions revealed no known resistance causing

mutations, however two unreported SNPs which result in amino acid substitutions (D414N and C277S) were observed. This is the first report of PPO resistance in *A. retroflexus* from the United States. Early and rapid identification of developing herbicide-resistant populations is essential for control and eradication.

Weedy *Amaranthus* spp. each possess unique morphological and genetic features beneficial for proliferation. Hybridization potential between *A. tuberculatus* and *A. albus* was determined through reciprocal crossing in a controlled environment. Progeny of each cross was collected separately and analyzed for hybrid frequency. A unidirectional hybridization frequency of 0.0046% was observed from *A. tuberculatus* to *A. albus*, as confirmed through molecular markers. No putative hybrids were observed from *A. tuberculatus* females. Interestingly, a significantly skewed male:female ratio from 1:1 was observed (87% female, P-value < 0.001). DNA content analysis of the first back cross population indicated polyploidization may be required for hybridization. Gene flow between two species allows highly beneficial traits, such as herbicide resistance, to spread outside a given species, influencing control options. Quantification of gene flow is necessary for predictive modelling of the spread of herbicide resistance within the *Amaranthus* genus.

Full plant regeneration is a necessary protocol for many aspects of basic science, including the analysis of gene function. The development of plant regeneration procedures relies on variation of plant hormones and media conditions. Regeneration of callus from leaf discs was consistently achieved utilizing a Murashige and Skoog media (MS) supplemented with the auxin indole-3-acetic acid (IAA) at 0.9 mg L^{-1} and the cytokinin trans-zeatin-riboside (zeatin) at 2.8 mg L^{-1}. Root regeneration was consistently achieved from callus utilizing a MS media supplemented with 4.2 mg L^{-1} zeatin in the absence of IAA. Utilizing a linear dilution series, zeatin was

determined to be unable to elicit shoot organogenesis under tested conditions. Continued utilization of dilution series of alternative cytokinins, such as 6-benzylaminopurine and thidiazuron, is expected to increase the probability of identifying optimal conditions for shoot organogenesis.

As weeds evolve within existing management systems, both surveillance and new advances are required to protect producers. An understanding of how weed species and herbicide-resistance traits move geographically can advise management practices to limit their spread. Quantifying gene flow between species can assist in predicting the next species to develop resistance to a given herbicide, facilitating surveillance and early detection. Characterizing new herbicide-resistance traits is required for rapid and high-throughput diagnosis of resistance in the absence of phenotyping. Finally, the development of new technologies, both to study key driver weeds and to control them, provide a future where, while existing management practices may break, new practices are available to take their place.

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CHAPTER 1: INTRODUCTION

A major source of yield loss within agricultural systems can be attributed to weed pressure. In 2012, the United States Department of Agriculture's National Agricultural Statistics Service (USDA-NASS) attributed 39% of yield losses reported in soybean to be caused by weeds, while plant disease was attributed to 4% (USDA-NASS 2014). Weed control in production agriculture has increasingly relied on herbicides. In 2015, herbicides were applied to 96% of planted soybean acres in the United States (USDA-NASS 2016). Intriguingly, the two most applied herbicide formulations were two salts of a single herbicide active, glyphosate. The potassium salt was applied to 55% of planted acres, while the isopropylamine salt was applied to 30% of planted acres. In 2012, farmers reported a decline in the sole reliance on glyphosate for weed control due to growing resistance issues; however, glyphosate was applied either alone or in combination on nearly all planted acres at that time point (USDA-NASS 2012).

Herbicide resistance has continued to grow in species abundance since the release of herbicides (Heap 2014). Not only has species abundance increased, the distribution of herbicide-resistance traits within key driver weed species have increased over time. Recent herbicide-resistance surveys conducted throughout the Midwest indicate that resistance to glyphosate has become widespread (Chatham et al 2014; Scultz et al 2014; Vieira et al 2018). Sole reliance on SoA where resistance is prevalent may result in failure to control the target weeds, leading to yield losses. As observed by the USDA-NASS (2016), the preferred management practice appears to be to rely on additional SoA, to which there might not yet be resistance. Unfortunately, a key driver weed within the US corn-belt, *Amaranthus tuberculatus*, was recently documented to be resistant to its sixth SoA, resulting in limited control options in affected regions (Bernards et al 2012). Should herbicide resistance continue to both develop and

spread, tank-mixing additional herbicides may no longer provide adequate weed control to limit yield losses.

Both the discovery of new herbicide-resistance cases and management decisions made by farmers heavily rely on field scouting for weeds. Continued observation of weedy populations is required to observe changes characteristic of resistance. Large scale initiatives, such as state-wide surveys, are highly beneficial as a large number of distinct populations can be surveyed near-simultaneously, and the prevalence of resistance mechanisms, in addition to resistance phenotype, is often quantified (Chatham et al 2014; Scultz et al 2014; Vieira et al 2018). The selection and characterization of plants where no known resistance mechanisms are observed is key in the discovery of new herbicide-resistance mechanisms. Screening for these new mechanisms can be further incorporated into surveillance initiatives. A key limitation of these initiatives is the time, space, and labor requirement for phenotyping (Chatham et al 2014). Should all resistance mechanisms be accounted for within the screened population, high-throughput mechanism screening would be sufficient to quantify resistance prevalence. However, moving from a plant phenotyping screening approach to a resistance screening approach may prevent new mechanisms of resistance from being observed until loss of control is observed at a field level.

The spread of herbicide resistance has been fairly well documented. The rate at which herbicide resistance traits move throughout a region can depend on multiple factors: initial resistance frequency, rate of mutation, seed movement, gene flow, inheritance of resistance, and fitness penalties associated with resistance and susceptibility (Diggle and Neve 2001). Perhaps the easiest factor to control, from a producer's perspective, is the movement of seed. Cleaning equipment and using certified seed are ideal ways to limit the movement of herbicide-resistance

traits from their source (Heap 2014). Gene flow, both within and between populations and species, can only be controlled through prevention of flowering. The initial frequency, rate of mutation, inheritance and fitness penalties associated with resistance are largely out of the control of farmers; however, quantifying these parameters is necessary for accurate modelling (Diggle and Neve 2001). Quantification of these factors is required to slow the rate of movement of herbicide-resistance traits and herbicide-resistant biotypes and species throughout a given region.

Attribution

In chapter two, I conducted all of the described research and wrote the first draft of the manuscript. Co-author Dr. Patrick Tranel co-designed the project and contributed to interpretation of the data and finalizing the manuscript. The manuscript was published in Crop Science. In chapter three, the development and validation of the seed extraction procedure was conducted by co-authors Diane Plewa, Elizabeth Phillippi, and Suzanne Bissonnette. I conducted the remaining described research and wrote the first draft of the manuscript. Co-author Dr. Patrick Tranel co-designed the project and contributed to the interpretation of the data and finalized the manuscript. The manuscript was published in Pest Management Science. In chapter four, the population collection was conducted by Dr. Mark Loux. The described research was equally conducted by myself and Alvaro Larran. Dr. Patrick Tranel co-designed the project and contributed to the interpretation of the data. In chapter five, accessions were provided by Dr. Mark Loux. I conducted the described research. Dr. Patrick Tranel co-designed the project and contributed to the interpretation of the data. In chapter six, putative hybrid populations were generated by Laura Chatham. Flow cytometry was conducted by Danielle McCormick. I conducted the remaining described research. Drs. Patrick Tranel co-designed the project and

contributed to the interpretation of the data. Dr. Lane Rayburn contributed to the interpretation of the data. In appendix A, I conducted the described research. Dr. Tranel co-designed the project and contributed to the interpretation of the data.

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CHAPTER 2: IDENTIFICATION AND VALIDATION OF *AMARANTHUS* SPECIES SPECIFIC SNPS WITHIN THE *ITS* REGION: APPLICATIONS IN QUANTITATIVE SPECIES IDENTIFICATION[1]

ABSTRACT

The *Amaranthus* genus consists of as many as 70 species, many with similar morphology. The development and validation of a DNA barcode specific to key amaranths would aid in plant identification. These barcodes can be used to develop assays for single-species identification, critical for species surveillance and contaminant screening. A reference panel of 75 internal transcribed spacer (ITS) sequences from the National Center for Biotechnology Information (NCBI) across 11 *Amaranthus* spp. was analyzed for species-specific single-nucleotide polymorphisms (SNPs). Identified SNPs were validated using 92 accessions of an *Amaranthus* spp. diversity panel. Of the 75 investigated ITS sequences from NCBI, 13 were identified as potentially mislabeled. Phylogenetic analysis of ITS from the reference panel distinguished 9 of the 11 investigated species. Nine SNPs were validated as species specific. Five species were distinguished with single SNPs. To illustrate the utility of the ITS SNP analysis, a quantitative assay for *Amaranthus tuberculatus* (Moq.) Sauer identification was constructed targeting SNP 73G and validated using simulated population samples. Results from this research will aid in population screening for *A. tuberculatus*, and in the development of other quantitative detection assays for *Amaranthus*.

[1] This research has been previously published in Crop Sciences (58:304-311). The copyright owner has provided permission to reprint.

INTRODUCTION

The *Amaranthus* genus consists of as many as 70 species, including both weedy and cultivated species (Stetter and Schmid, 2017). In the Great Plains region, at least 10 *Amaranthus* species are commonly observed and considered troublesome weeds in agricultural production (Franssen et al., 2001). Each species possesses a geographical range, which may overlap, forming unique population structures throughout the region. For example, *Amaranthus palmeri* S. Watson is not naturalized in the Northern Great Plains region, whereas *Amaranthus retroflexus* L. is naturalized throughout the entire Great Plains region (USDA-ARS GRIN, 2016). The maintenance of geographic isolation of highly competitive *Amaranthus* species, such as *A. palmeri* and *Amaranthus tuberculatus* (Moq.) Sauer, is preferable. In 2016, *A. palmeri* introductions were confirmed in 48 counties in Iowa, in fields seeded to Conservation Reserve Program acres (Hartzler and Anderson, 2016). These introductions are a prime example of potential causes and consequences of the loss of regional isolation of competitive weed species.

Amaranthus species identification is necessary for early detection of introductions. Current diagnostic techniques allow the identification to the species level using both morphological and molecular techniques. Morphological diagnostics are based on mature plant morphology, such as floral morphology, flower placement, pollen morphology, and growth habit (Horak et al., 1994). Morphological diagnostics for seed and early seedling growth stages are unreliable or absent. Seed identification in the absence of mature plant morphology is unreliable (AOSA, 2016). Morphological diagnostics are reviewed in (Horak et al., 1994), and pollen grain diagnostics are reviewed in (Franssen et al., 2001).

A confounding factor in the identification of *Amaranthus* spp. is hybridization between species. The distributions of many amaranths overlap, allowing opportunities for hybridization to

occur in nature. Hybridization between *A. palmeri* and *Amaranthus spinosus* L. has been observed to transfer a glyphosate resistance trait (Gaines et al., 2012; Nandula et al., 2014). Unidirectional genetic exchange between *A. tuberculatus* and *Amaranthus hybridus* L. has been observed through amplified fragment length polymorphism genotyping (Trucco et al., 2009). Hybridization between *A. palmeri* and *A. tuberculatus* has been observed to transfer resistance to acetolactate synthase inhibitors under laboratory conditions (Wetzel et al., 1999b). Morphology cannot reliably distinguish hybrids from the parent species.

Several considerations make the use of morphological diagnostics to distinguish major *Amaranthus* spp. impractical. Both pollen and floral structure diagnostics require mature plants; however, weed control is often required before weeds reach maturity (Van Acker et al., 1993). Furthermore, training and equipment are required to correctly identify diagnostic characteristics. For dioecious species, the gender of a given plant can limit the available diagnostics. The development of molecular markers for species differentiation allows for rapid and accurate species identification (Wetzel et al., 1999a).

To date, several molecular assays have been developed for the identification of common subsets of the *Amaranthus* genus. A random amplification of polymorphic DNA (RAPD) method was developed for the differentiation of three grain amaranth species (Transue et al., 1993). A restriction fragment length polymorphism (RFLP) method using the internal transcribed spacer (ITS) (Wetzel et al., 1999a) was used to differentiate between nine common weedy amaranths. More recently, a polymerase chain reaction (PCR) method for the identification of seven common weedy amaranths was developed (Wright et al., 2016). Additionally, several molecular identification methods have been developed for single-species identification, including both cultivated and weedy amaranths (Park et al., 2014). Many of these methods, however, were

validated against a narrow range of species, often using populations from narrow geographic origins, which might not be representative of the species. Furthermore, these methods often require multistep procedures (e.g., PCR followed by restriction digest and gel electrophoreses) for the identification of a single plant. Scalability and throughput are major considerations in the development and implementation of a surveillance strategy. Although both training and equipment are required to implement a molecular-marker-based surveillance strategy, scalability and throughput are substantially greater than observed with a morphology-based surveillance strategy.

Previously, a new method for the identification of *A. palmeri* contamination in mixed seed lots was developed (Murphy et al., 2017) based on polymorphisms in the ITS region, which has proven to be a suitable barcoding region to differentiate *Amaranthus* spp. (Wetzel et al., 1999a; Waselkov, 2013). In the present study, we extend this work to further explore the viability of the ITS region as a DNA barcoding region for distinguishing additional *Amaranthus* species. Species-specific single-nucleotide polymorphisms (SNPs) and barcodes were validated using a diverse collection of *Amaranthus* spp., including multiple accessions of both cultivated and weedy species. To illustrate the utility of the barcodes, a molecular assay for the quantitative detection of *A. tuberculatus* was developed.

MATERIALS AND METHODS

Species-Specific SNP Identification

Ribosomal RNA sequence from *Amaranthus albus* L. (Genbank accession no. JF975853) was queried against the National Center for Biotechnology Information (NCBI) nucleotide database using blastn with default parameters, resulting in 90 hits within the *Amaranthus* genus

(Taxonomy ID: 3564). The sequences of species represented three or more times in this list were extracted, with the exception of *Amaranthus powellii* S. Watson, which was represented twice. These sequences, which came from 11 species, composed the reference panel. Predicted digest patterns based on an RFLP assay (Wetzel et al., 1999a) were used to assist in validation of species identity. Sequences were aligned using MAFFT 7.311, and species-specific SNPs were called using a custom script. Species-specific SNPs and barcodes composed of multiple diagnostic SNPs were subsequently identified. Phylogenetic analysis was conducted using dnaml (phylip-3.695) on default parameters and FigTree v1.4.3.

Plant Accessions

Accessions of *Amaranthus blitoides* S. Watson, *A. hybridus, A. palmeri, A. powellii, A. retroflexus,* and *A. tuberculatus* were obtained from University of Illinois in-house collections. Accessions of *A. albus, Amaranthus arenicola* I.M. Johnst., *Amaranthus blitum* L., and *Amaranthus hypochondriacus* L. were obtained from the Germplasm Resources Information Network (GRIN, Supplemental Table S2). At least five accessions of each species were included, based on species label. These accessions composed the validation panel. Seeds were grown under greenhouse conditions, as described previously (Patzoldt et al., 2006).

DNA Extraction

Biological triplicates of young leaf tissue were collected from one plant of each accession. Leaf DNA extraction followed a cetyl trimethylammonium bromide (CTAB) procedure (Doyle and Doyle, 1990). DNA was quantified with Nanodrop (Thermo Fisher Scientific) and diluted to 100 ng μL−1.

DNA Sequencing

Subsets of accessions, up to five per species, were selected for DNA sequencing and analysis. The ITS region was amplified as described previously (Wetzel et al., 1999a) and purified using the E.Z.N.A. cycle-pure kit (Omega Bio-tek). Purified product was used for Sanger sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

Species-Specific SNP Validation

DNA sequencing chromatographs were manually curated, and SNP calling was conducted as described in SNP identification. Previously identified loci (from the reference panel) were investigated to validate candidate SNPs for species identification.

qPCR

All accessions included in the validation panel were used for quantitative PCR (qPCR) validation of an *A. tuberculatus* marker. Primers for *A. tuberculatus* identification (forward, 5′-GCCTTACGGACGAGCTGATG-3′, and reverse, 5′-CCGTTGCCGAGAGTCGTTC-3′) were designed using an *A. tuberculatus*-specific SNP. Each qPCR reaction consisted of 5 µL iTaq Universal SYBR Green Supermix (Bio-Rad), 1 µL forward primer and 1 µL reverse primer (each 10 µM), 1 µL template DNA, and 2 µL water. A previously identified qPCR control gene, CPS, was included as a single copy control (Ma et al., 2013). The qPCR thermoprofile was as follows: 95°C for 5 min and 35 cycles of 59 °C for 1 min and 95°C for 15 s, immediately followed by a melt curve analysis for quality control of positive samples (Taylor et al., 2010). Technical duplicates were used to minimize error. An arbitrarily chosen *A. tuberculatus* accession was used for a six-step, 1:4 dilution series of the sample with water. A five-step, 1:3 mixture series of *A.*

palmeri and *A. tuberculatus* DNA was conducted to demonstrate the application of these markers to a mixed population sample.

Statistical Analysis

K-mer cluster analysis ('cluster' R package) was used to differentiate accessions (k = 2). The ΔΔcycle threshold method was used to analyze mixture series (Schmittgen and Livak, 2008).

RESULTS AND DISCUSSION

The geographic origins of accessions of the reference panel were skewed to China and India (Figure 2.1). Ideally, the geographic origins of the accessions would be spread across an increased number of continents. The geographic origins of accessions of the combined reference and validation panel show a similar representation of accessions from China and the United States. The validation panel included an increased diversity of countries to ensure that species-specific SNPs were not region specific, but reflective of the greater diversity within the species (Table 2.1).

Analysis of the reference panel using both phylogenetic clustering and predicted restriction digest patterns revealed that 13 of 75 sequences are potentially mislabeled (Table 2.2). In the absence of morphological information, the true identity of these accessions cannot be confirmed. Furthermore, the supporting information for these sequences is largely unpublished, preventing follow up. Although alternative molecular diagnostic methods are available (Wright et al., 2016), additional sequence information is required, preventing their use. Additionally, these assays have not been validated across the range of species investigated in this study. Restriction digest patterns have been previously demonstrated to be diagnostic for amaranth

species identification (Wetzel et al., 1999a). Predicted restriction digest analysis of these suspect sequences supports the proposed relabeling (Table 2.3). However, as not all species investigated in this study can be identified with the RFLP assay, additional incorrect labeling is possible. Additionally, some of the accessions yielded predicted RFLP patterns that were not previously reported. Therefore, phylogenetic clustering was used to reduce the potential for mislabeling for all species. A candidate identification of the potentially mislabeled sequences is proposed and used in subsequent sections (Table 2.2).

Several species included in the reference panel were outside the species range covered by the RFLP assay: *A. blitum*, *A. hypochondriacus*, *Amaranthus tricolor* L., and *Amaranthus viridis* L. For these species, a consistent predicted digest pattern was observed, diagnostic of *A. tuberculatus*, with the exception of *A. hypochondriacus*, which was identified as *A. hybridus* (Table 2.3). This highlights a major limitation of existing assays, where a narrow species range used for assay development can lead to false identification.

Phylogenetic analysis distinguished 11 of the 12 species composing the reference panel (Figure 2.2), whereas the restriction digest array was able to distinguish 8 of the 11 species (Table 2.4). Most notably, phylogenetic analysis distinguished between *A. tricolor* and *A. blitum*, two cultivated leafy vegetable amaranths. Distinguishing between cultivated and weedy species allows for germplasm conservation (Ray and Roy, 2009). However, *A. hypochondriacus*, a cultivated grain species, was indistinguishable from *A. hybridus* (Chan and Sun, 1997).

Several instances of within-species ITS sequence variation was observed within the reference panel for some species, most notably within the A. hybridus cluster. However, in some species, such as A. palmeri, no species variation was observed (Figure 2.2).

A recent analysis of the phylogenetic relationships within the Amaranthus genus using genotype-by-sequencing (GBS) data was conducted (Stetter and Schmid, 2017). A comparison of the species groupings (Figure 2.2) and GBS data revealed many similarities. Amaranthus retroflexus and A. powellii are closely related but distinguishable. A similar trend was noticed for the following species groups: *A. spinosus* and *A. palmeri*, *A. blitum* and *A. tricolor*, and *A. blitoides* and *A. albus*. These observations suggest that the ITS region is reflective of the divergence that has occurred through the speciation of the *Amaranthus* genus until the recent selection of *A. hypochondriacus* and support the use of the ITS region for species identification within the *Amaranthus* genus. Similar species clustering was observed previously by Waselkov (2013), who conducted phylogenetic analysis of 57 *Amaranthus* spp. using the ITS, as well as other regions, although she used few accessions per species.

A SNP array was constructed from the alignment of the reference sequence panel. A total of 44 polymorphic nucleotides were observed, 13 of which contained a SNP specific to an individual species, and 20 of which contained within-species variation (data not shown). Sequence information from the validation panel was used to confirm usefulness of the SNPs identified from the reference panel. Upon sequencing validation, within-species variation was observed in all but nine SNPs. Five species could be differentiated from all other species by a single SNP: 73A is diagnostic of *A. retroflexus*, 73G of *A. tuberculatus*, both 28A and 108A of *A. palmeri*, 692A of *A. tricolor*, and 83T of *A. blitum* (Table 2.4). Species-specific SNPs and barcodes were validated for nine individual species and the *A. hybridus* + *A. hypochondriacus* cluster (Table 2.4). An overabundance of mislabeled *A. blitoides* accessions, based on the RFLP assay (Wetzel et al., 1999a) and morphological characteristics, in the reference and validation panels prevented SNP calling for this species. Within the validation panel, accessions of *A.*

arenicola were included. This species was not included in the reference panel due to lack of available sequences. No sequence variation was observed between *A. arenicola* and *A. tuberculatus* sequences. Interestingly, previously conducted phylogenies had grouped *A. arenicola* closer to *A. palmeri*, rather than to *A. tuberculatus* (Stetter and Schmid, 2017). The similarity of the ITS region between these species could simply be due to random chance, rather than a demonstration of a speciation event. Species-specific barcodes were identified for nine species and one species cluster.

Using qPCR, SNP 73G, specific to *A. tuberculatus*, was validated as distinct from the species investigated in the reference panel (Figure 2.3). However, *A. arenicola* accessions were incorrectly identified as *A. tuberculatus*, a result similar to the sequence analysis. This result demonstrates the dangers of including species that were not considered in the development of an assay. *Amaranthus arenicola* accessions were included in the PCR validation to determine the behavior of the developed assay on accessions not included in species-specific SNP identification. Since *A. arenicola* was not present within the reference panel, its behavior at loci believed to be specific for *A. tuberculatus* cannot be predicted. The SNP 108A, specific to *A. palmeri*, was previously validated (Murphy et al., 2017).

The sensitivity of the *A. tuberculatus*-specific marker was determined using a dilution series. A high correlation between cycle threshold and DNA concentration was observed (Figure 2.4A). A mixture series of *A. tuberculatus* and *A. palmeri* DNA was used to demonstrate the application of this marker to population surveillance (Figure 2.4B). At a dilution of 1:81 *A. tuberculatus* DNA with *A. palmeri* DNA, the ΔΔcycle threshold value was distinguishable from all other tested species, with the exception of *A. arenicola*. Previously, the mixed DNA method was demonstrated to be comparable with seed lot extracted DNA samples (Murphy et al., 2017),

indicating the developed *A. tuberculatus* markers can be applied to seed lot screening. Theoretically, the DNA barcode itself could be used as a surveillance tool for bulked population samples. Next-generation sequencing of the 5′ ITS region (150 bp) would be sufficient to capture seven species-specific barcodes and two species clusters: *A. hybridus* + *A. hypochondriacus* and *A. viridis* + *A. tricolor* clusters. Additionally, a candidate species cluster of *A. tuberculatus* and *A. arenicola* was observed; however, this observation lacks support in the available literature (Stetter and Schmid, 2017). Sequencing depth of this region in excess of the number of bulked samples would provide an approximation of each species' abundance within the sample. Changes over time, as well as the presence of previously unseen species, would be observable through this method.

This study highlights concerns when developing species-specific assays. A wide geographic range should be surveyed. Ideally, the geographic range of the investigated species should be represented to prevent region-specific SNPs from being viewed as diagnostic. Previous assays have been limited by few accessions per species tested (Wright et al., 2016). We attribute the decrease in informative loci observed in our study when comparing the reference with the validation panel to this effect. In regards to our study, using the wide geographical range encompassing both the reference and validation panel, combined with our sample size, was critical in the identification of species-specific SNPs. Additionally, public availability of germplasm from which published and unpublished sequence information was obtained would increase the ease and certainty of the development of species-specific DNA barcodes. In this study, 13 sequences from the reference panel are believed to be mislabeled. This claim is supported by predicted digest patterns previously identified as diagnostic (Wetzel et al., 1999a) and phylogenetic clustering conducted in this study. The validation, with morphologically

characterized accessions, of SNPs observed on relabeling these sequences offers further evidence in support of their relabeling. Should the accessions used to generate these suspect sequences be publically available, independent morphological characterization would aid in proper identification.

A key limiting factor in the development of molecular-marker-based species identification is the breadth of the species panel used to construct the assay. Species that were not considered in the design of the assay will have unpredictable behavior. *Amaranthus arenicola* was observed to be identified as *A. tuberculatus* using the markers developed in this paper. Previously, *A. arenicola* was observed to be correctly identified as "not *A. palmeri*" (Murphy et al., 2017). These observations demonstrate the unpredictable nature of unknown species when developing species-specific markers.

Proper species identification is a first step towards minimizing the spread of noxious weedy species. Despite the limitations just described, DNA barcoding and marker-based approaches enable high throughput and automation, which are required for largescale surveillance and management initiatives. These initiatives are necessary to maintain geographic isolation boundaries of key agricultural weeds, such as *A. palmeri*. Recently, a breakdown of the geographic isolation of *A. palmeri* was observed in Iowa, where introductions have now been confirmed in 48 counties (Hartzler and Anderson, 2016). *Amaranthus palmeri* is a highly competitive species (Bensch et al., 2003), in which multiple herbicide resistances have been documented (Heap, 2017). Our research presented here highlights the utility—and caveats—of using ITS-based barcodes for identification of *Amaranthus* species.

ACKNOWLEDGEMENT

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TABLES AND FIGURES

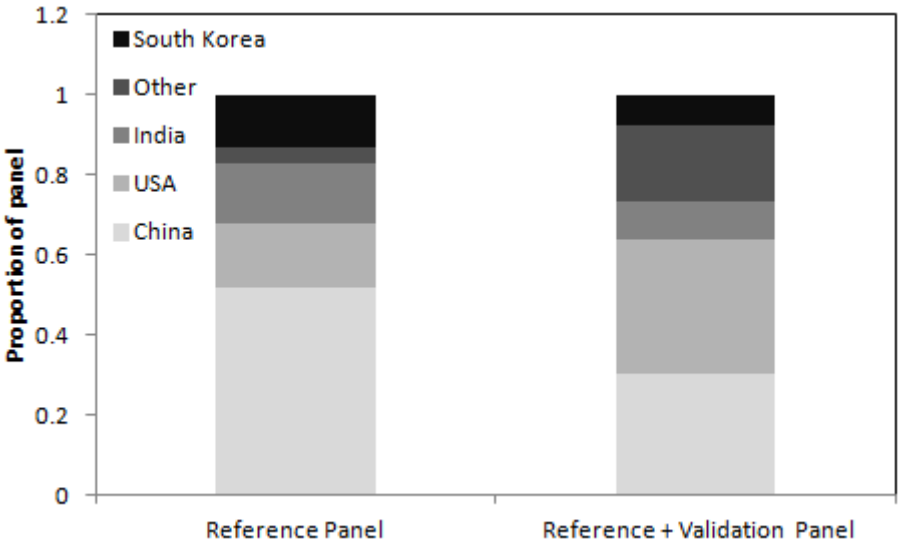

Figure 2.1. Geographic origins of investigated accessions. Country label "other" are accessions from countries not otherwise labeled.

Table 2.1. List of validation panel accessions. All accessions were included in qPCR analysis; a "Y" in the sequencing column indicates accession from which *ITS* was sequenced.

Accession	Species	Country	Source	Accession	Sequencing	qPCR
1	*A. powellii*	USA	UIUC	234	Y	Y
2	*A. powellii*	USA	UIUC	235	Y	Y
3	*A. powellii*	USA	UIUC	237	Y	Y
4	*A. powellii*	USA	UIUC	239	N	Y
5	*A. powellii*	USA	UIUC	240	Y	Y
6	*A. powellii*	USA	UIUC	241	Y	Y
7	*A. retroflexus*	Canada	UIUC	242	N	Y
8	*A. powellii*	Canada	UIUC	243	N	Y
9	*A. retroflexus*	USA	UIUC	244	N	Y
10	*A. powellii*	Canada	UIUC	246	N	Y
11	*A. hybridus*	USA	UIUC	165	N	Y
12	*A. hybridus*	USA	UIUC	68	N	Y
13	*A. hybridus*	Canada	UIUC	172	N	Y
14	*A. hybridus*	Canada	UIUC	173	Y	Y
15	*A. hybridus*	USA	UIUC	176	Y	Y
16	*A. hybridus*	USA	UIUC	177	N	Y
17	*A. hybridus*	USA	UIUC	178	Y	Y
18	*A. hybridus*	USA	UIUC	182	Y	Y
19	*A. hybridus*	USA	UIUC	183	N	Y

Table 2.1. (Continued)

Accession	Species	Country	Source	Accession	Sequencing	qPCR
20	*A. hybridus*	USA	UIUC	184	N	Y
21	*A. hybridus*	Canada	UIUC	206	N	Y
23	*A. albus*	USA	UIUC	209	N	Y
25	*A. blitoides*	USA	UIUC	211	Y	Y
27	*A. hybridus*	USA	UIUC	213	N	Y
28	*A. hybridus*	USA	UIUC	215	N	Y
30	*A. hybridus*	USA	UIUC	219	N	Y
31	*A. retroflexus*	USA	UIUC	72	Y	Y
32	*A. retroflexus*	USA	UIUC	287	Y	Y
33	*A. powellii*	Canada	UIUC	296	N	Y
34	*A. retroflexus*	Canada	UIUC	70	N	Y
35	*A. retroflexus*	USA	UIUC	73	Y	Y
36	*A. powellii*	USA	UIUC	74	N	Y
37	*A. hybridus*	USA	UIUC	75	N	Y
38	*A. retroflexus*	USA	UIUC	78	Y	Y
39	*A. retroflexus*	USA	UIUC	79	N	Y
40	*A. retroflexus*	USA	UIUC	80	N	Y
41	*A. retroflexus*	USA	UIUC	261	N	Y
42	*A. palmeri*	USA	UIUC	263	N	Y
43	*A. palmeri*	USA	UIUC	265	N	Y
44	*A. palmeri*	USA	UIUC	281	Y	Y

Table 2.1. (Continued)

Accession	Species	Country	Source	Accession	Sequencing	qPCR
45	*A. palmeri*	USA	UIUC	283	Y	Y
46	*A. palmeri*	USA	UIUC	284	Y	Y
47	*A. palmeri*	USA	UIUC	292	N	Y
48	*A. palmeri*	USA	UIUC	291	N	Y
49	*A. palmeri*	USA	UIUC	288	N	Y
50	*A. palmeri*	USA	UIUC	259	N	Y
51	*A. palmeri*	USA	UIUC	130	N	Y
52	*A. palmeri*	USA	UIUC	131	N	Y
53	*A. palmeri*	USA	UIUC	132	N	Y
54	*A. palmeri*	USA	UIUC	133	N	Y
55	*A. tricolor*	USA	UIUC	34	Y	Y
56	*A. tricolor*	Taiwan	GRIN	RRC42	Y	Y
58	*A. tuberculatus*	USA	UIUC	142	Y	Y
59	*A. hybridus*	USA	UIUC	141	Y	Y
60	*A. palmeri*	USA	UIUC	129	Y	Y
62	*A. viridis*	Argentina	GRIN	RRC 1381	Y	Y
63	*A. tricolor*	Congo	GRIN	RRC26	Y	Y
64	*A. tricolor*	India	GRIN	RRC29	Y	Y
65	*A. tricolor*	Taiwan	GRIN	RRC42	Y	Y

Table 2.1. (Continued)

Accession	Species	Country	Source	Accession	Sequencing	qPCR
66	*A. hypochondriacus*	Dominican Republic	GRIN	RRC95	Y	Y
67	*A. tricolor*	Malaysia	GRIN	RRC107	Y	Y
68	*A. spinosus*	Indonesia	GRIN	RRC108C	Y	Y
69	*A. tricolor*	Nepal	GRIN	RRC158	Y	Y
70	*A. hypochondriacus*	Mexico	GRIN	GN855	N	Y
71	*A. blitum*	Hong Kong	GRIN	RRC313	Y	Y
72	*A. viridis*	Seychelles	GRIN	RRC316	N	Y
73	*A. hypochondriacus*	USA	GRIN	RRC383	Y	Y
74	*A. albus*	Puerto Rico	GRIN	RRC392	Y	Y
75	*A. viridis*	Philippines	GRIN	RRC864	N	Y
76	*A. spinosus*	Zambia	GRIN	ZM2771	N	Y
77	*A. arenicola*	USA	GRIN	A	N	Y
78	*A. arenicola*	USA	GRIN	D	Y	Y
79	*A. blitum*	Switzerland	GRIN	AMES 2497	Y	Y
80	*A. arenicola*	USA	GRIN	POP 57	Y	Y
81	*A. hypochondriacus*	China	GRIN	RRC101C	N	Y

Table 2.1. (Continued)

Accession	Species	Country	Source	Accession	Sequencing	qPCR
82	*A. spinosus*	Indonesia	GRIN	RRC114	N	Y
83	*A. spinosus*	Thailand	GRIN	RRC115	Y	Y
84	*A. hypochondriacus*	Nepal	GRIN	RRC124	Y	Y
85	*A. blitum*	Hong Kong	GRIN	RRC298	Y	Y
86	*A. albus*	USA	GRIN	DB200125	Y	Y
87	*A. blitum*	USA	GRIN	DB200126	Y	Y
88	*A. spinosus*	USA	GRIN	DB200130	Y	Y
89	*A. arenicola*	Canada	GRIN	AMA65	Y	Y
90	*A. tuberculatus*	Spain	GRIN	AMA96	Y	Y
91	*A. albus*	Portugal	GRIN	INDEX SEMINUM 2	Y	Y
92	*A. blitum*	Brazil	GRIN	CPAC96-19	Y	Y
93	*A. viridis*	USA	GRIN	DB8913	Y	Y
94	*A. albus*	USA	GRIN	AMES 18499	Y	Y
95	*A. arenicola*	Mexico	GRIN	LP146	Y	Y
96	*A. arenicola*	USA	GRIN	DB2008061	Y	Y
97	*A. tuberculatus*	USA	UIUC	05x31	Y	Y
98	*A. tuberculatus*	USA	UIUC	2A3	Y	Y

Table 2.2. Potentially mislabelled sequences within reference panel.

Genbank	Provided ID	Proposed ID	Justification
KU310614	*A. tricolor*	*A. hybridus*	Phylogenetic clustering
KF493807	*A. palmeri*	*A. hybridus*	Phylogenetic clustering; restriction digest array
JF975859	*A. retroflexus*	*A. hybridus*	Phylogenetic clustering; restriction digest array
KF493795	*A. retroflexus*	*A. hybridus*	Phylogenetic clustering; restriction digest array
KF493799	*A. retroflexus*	*A. tuberculatus*	Phylogenetic clustering
KF493802	*A. retroflexus*	*A. tuberculatus*	Phylogenetic clustering
KU310612	*A. retroflexus*	*A. hybridus*	Phylogenetic clustering; restriction digest array
L78085	*A. retroflexus*	*A. hybridus*	Phylogenetic clustering; restriction digest array
KP178673	*A. hybridus*	*A. powellii*	Phylogenetic clustering; restriction digest array
AF210908	*A. spinosus*	*A. palmeri*	Phylogenetic clustering
KF493804	*A. spinosus*	*A. tuberculatus*	Phylogenetic clustering; restriction digest array
KP318852	*A. albus*	*A. blitoides*	Phylogenetic clustering; restriction digest array
KF493781	*A. blitoides*	*A. tuberculatus*	Phylogenetic clustering; restriction digest array

Bolded entries have truncated 3’ region of read, preventing identification with restriction digest array.

Table 2.3. List of reference panel *ITS* sequences. Predicted identities are based on restriction enzyme digest patterns. NaN indicates a digestion pattern not previously associated with a particular species.

SI Table 2. List of reference panel *ITS* sequences. Predicted identities are based on restriction enzyme digest patterns. NaN indicates a digestion pattern not previously associated with a particular species.

Sequence	Species	Submitting Country	AvaI	BsaAI	DdeI	HaeIII	XhoI	Predicted Identity
KF493787.1	*A. viridis*	China	+	-	+	-	-	*A. tuberculatus*
KF493791.1	*A. viridis*	China	+	-	+	-	-	*A. tuberculatus*
KF493796.1	*A. viridis*	China	+	-	+	-	-	*A. tuberculatus*
KJ004298.1	*A. viridis*	Saudi Arabia	+	-	+	-	-	*A. tuberculatus*
KJ004300.1	*A. viridis*	Saudi Arabia	+	-	+	-	-	*A. tuberculatus*
KP318860.1	*A. viridis*	India	+	-	+	-	-	*A. tuberculatus*
KX090199.1	*A. viridis*	India	+	-	+	-	-	*A. tuberculatus*
AF210917.1	*A. hypochondriacus*	China	+	-	+	+	-	*A. hybridus*
KU310613.1	*A. hypochondriacus*	India	+	-	+	+	-	*A. hybridus*
KU310615.1	*A. hypochondriacus*	India	+	-	+	+	-	*A. hybridus*
KF385438.1	*A. palmeri*	China	+	+	-	-	-	*A. palmeri*
KF493782.1	*A. palmeri*	China	+	+	+	-	-	NaN
KF493784.1	*A. palmeri*	China	+	+	+	-	-	NaN
KF493786.1	*A. palmeri*	China	+	+	-	-	-	*A. palmeri*
KF493788.1	*A. palmeri*	China	+	+	+	-	-	NaN
KF493803.1	*A. palmeri*	China	+	+	+	-	-	NaN
KF493807.1	*A. palmeri*	China	+	-	+	+	-	*A. hybridus*
KM438060.1	*A. palmeri*	USA	+	+	-	-	-	*A. palmeri*
KM438061.1	*A. palmeri*	USA	+	+	-	-	-	*A. palmeri*

Table 2.3. (Continued)

Sequence	Species	Submitting Country	AvaI	BsaAI	DdeI	HaeIII	XhoI	Predicted Identity
KM438062.1	*A. palmeri*	USA	+	+	-	-	-	*A. palmeri*
KM438063.1	*A. palmeri*	USA	+	+	-	-	-	*A. palmeri*
KM438064.1	*A. palmeri*	USA	+	+	-	-	-	*A. palmeri*
KM438065.1	*A. palmeri*	USA	+	+	-	-	-	*A. palmeri*
KP318856.1	*A. palmeri*	India	+	+	-	-	-	*A. palmeri*
KF493778.1	*A. powellii*	China	+	-	+	+	-	*A. hybridus*
KP318861.1	*A. powellii*	India	+	-	-	+	-	*A. powellii*
KF493777.1	*A. blitum*	China	+	-	+	-	-	*A. tuberculatus*
KF493783.1	*A. blitum*	China	+	-	+	-	-	*A. tuberculatus*
KF493794.1	*A. blitum*	China	+	-	+	-	-	*A. tuberculatus*
KP318854.1	*A. blitum*	India	+	-	+	-	-	*A. tuberculatus*
KX090192.1	*A. blitum*	India	+	-	+	-	-	*A. tuberculatus*
AF210906.1	*A. retroflexus*	China	+	-	-	-	-	*A. retroflexus*
JF975859.1	*A. retroflexus*	China	+	-	+	+	-	*A. hybridus*
JF975860.1	*A. retroflexus*	China	+	-	-	-	-	*A. retroflexus*
JF975861.1	*A. retroflexus*	China	+	-	-	-	-	*A. retroflexus*
KF493795.1	*A. retroflexus*	China	+	-	+	+	-	*A. hybridus*
KF493799.1	*A. retroflexus*	China	+	-	+	-	-	*A. tuberculatus*
KF493802.1	*A. retroflexus*	China	+	-	+	-	-	*A. tuberculatus*
KF493805.1	*A. retroflexus*	China	+	-	-	-	-	*A. retroflexus*
KP318858.1	*A. retroflexus*	India	+	-	-	-	-	*A. retroflexus*
KU310612.1	*A. retroflexus*	India	+	-	+	+	-	*A. hybridus*
L78085.1	*A. retroflexus*	USA	+	-	+	+	-	*A. hybridus*
AF210910.1	*A. hybridus*	China	+	-	+	+	-	*A. hybridus*
DQ005960.1	*A. hybridus*	USA	+	-	+	+	-	*A. hybridus*
EF590750.1	*A. hybridus*	USA	+	-	+	-	-	*A. tuberculatus*
JF975857.1	*A. hybridus*	China	+	-	+	+	-	*A. hybridus*
KF493798.1	*A. hybridus*	China	+	-	+	+	-	*A. hybridus*

Table 2.3. (Continued)

Sequence	Species	Submitting Country	AvaI	BsaAI	DdeI	HaeIII	XhoI	Predicted Identity
KP178673.1	*A. hybridus*	India	+	-	-	+	-	*A. powellii*
KP318855.1	*A. hybridus*	India	+	-	+	+	-	*A. hybridus*
KP318857.1	*A. hybridus*	India	+	-	+	+	-	*A. hybridus*
KP658380.1	*A. hybridus*	India	+	-	+	+	-	*A. hybridus*
KR150154.1	*A. hybridus*	Sweden	+	-	+	+	-	*A. hybridus*
KX090195.1	*A. hybridus*	India	+	-	+	+	-	*A. hybridus*
KX090196.1	*A. hybridus*	India	+	-	+	+	-	*A. hybridus*
AF210908.1	*A. spinosus*	China	+	+	-	-	-	*A. palmeri*
AY174420.1	*A. spinosus*	USA	-	-	-	-	-	NaN
DQ005961.1	*A. spinosus*	USA	+	+	-	-	+	*A. spinosus*
EF590751.1	*A. spinosus*	USA	-	-	-	-	-	NaN
JF975863.1	*A. spinosus*	China	+	+	-	-	+	*A. spinosus*
KF493789.1	*A. spinosus*	China	+	+	+	-	+	NaN
KF493804.1	*A. spinosus*	China	+	-	+	-	-	*A. tuberculatus*
KP318859.1	*A. spinosus*	India	+	+	-	-	+	*A. spinosus*
KX090197.1	*A. spinosus*	India	+	+	+	-	+	NaN
AF210915.1	*A. tricolor*	China	+	-	+	-	-	*A. tuberculatus*
JF975871.1	*A. tricolor*	China	+	-	+	-	-	*A. tuberculatus*
KF385439.1	*A. tricolor*	China	+	-	+	-	-	*A. tuberculatus*
KU310614.1	*A. tricolor*	India	+	-	+	+	-	*A. hybridus*
KX090198.1	*A. tricolor*	India	+	-	+	-	-	*A. tuberculatus*
AF210918.1	*A. albus*	China	+	-	-	-	+	*A. albus*
JF975853.1	*A. albus*	China	+	-	-	-	+	*A. albus*
KP318852.1	*A. albus*	India	+	-	-	+	+	*A. blitoides*
KF385437.1	*A. tuberculatus*	China	+	-	+	-	-	*A. tuberculatus*
KF493781.1	*A. tuberculatus*	China	+	-	+	+	+	NaN
KF493793.1	*A. tuberculatus*	China	+	-	+	-	-	*A. tuberculatus*
KF493806.1	*A. tuberculatus*	China	+	-	+	-	-	*A. tuberculatus*

Table 2.4. Validated *Amaranthus* species-specific SNPs. In some cases, a single SNP is sufficient to distinguish a species; in others, two SNPs are needed. Numbers refer to nucleotide position within *ITS*. Within-species polymorphisms are indicated by Y (C or T), S (G or C), R (A or G), and M (A or C).

Species	28	58	63	69	73	83	108	141	607	692
A. hybridus	T	A	T	C	**A**	C	C	**C**	T	T
A. hypochondriacus	T	A	T	C	**A**	C	C	**C**	T	T
A. retroflexus	T	A	T	C	**T**	C	C	T	T	T
A. powellii	T	A	T	C	**A**	C	C	**T**	T	T
A. tuberculatus	T	A	Y	S	**G**	C	C	S	Y	T
A. viridis	T	**G**	T	**C**	-	**C**	C	C	T	**T**
A. tricolor	T	R	T	C	-	C	C	C	T	**C**
A. blitum	T	**G**	T	**T**	-	**T**	C	C	Y	T
A. palmeri	**A**	A	T	C	-	C	**A**	G	A	T
A. spinosus	T	A	T	C	-	C	**C**	S	**A**	T
A. albus	T	A	**C**	C	**-**	C	C	M	T	-

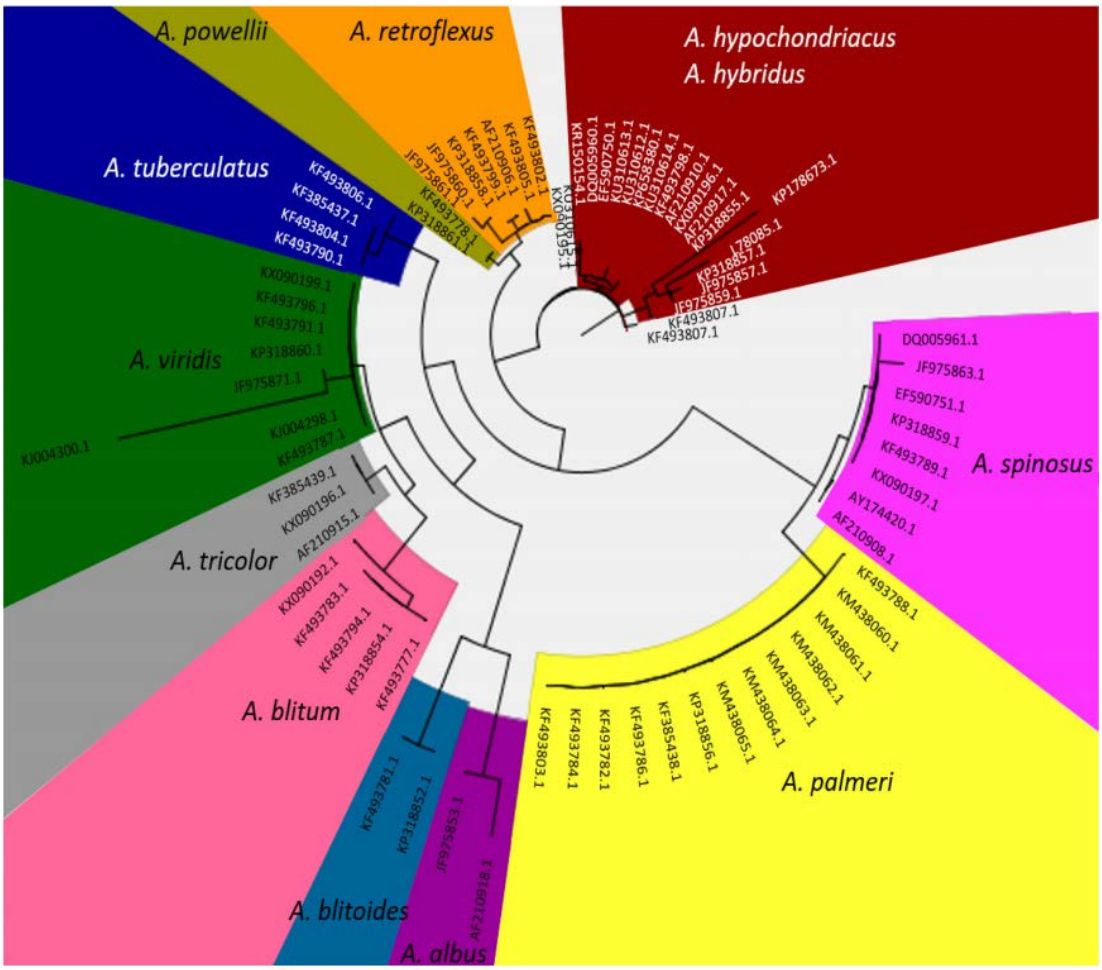

Figure 2.2. Radial phylogenetic tree of reference Amaranth sequences. Color coding is based on species identity as determined by RFLP assay (Wetzel et al., 1999a) where applicable. Species not covered in the RFLP assay were determined through phylogenetic analysis. Uncolored sequences were truncated, preventing proper placement, highlighting the necessity of the complete 5' *ITS* region for identification.

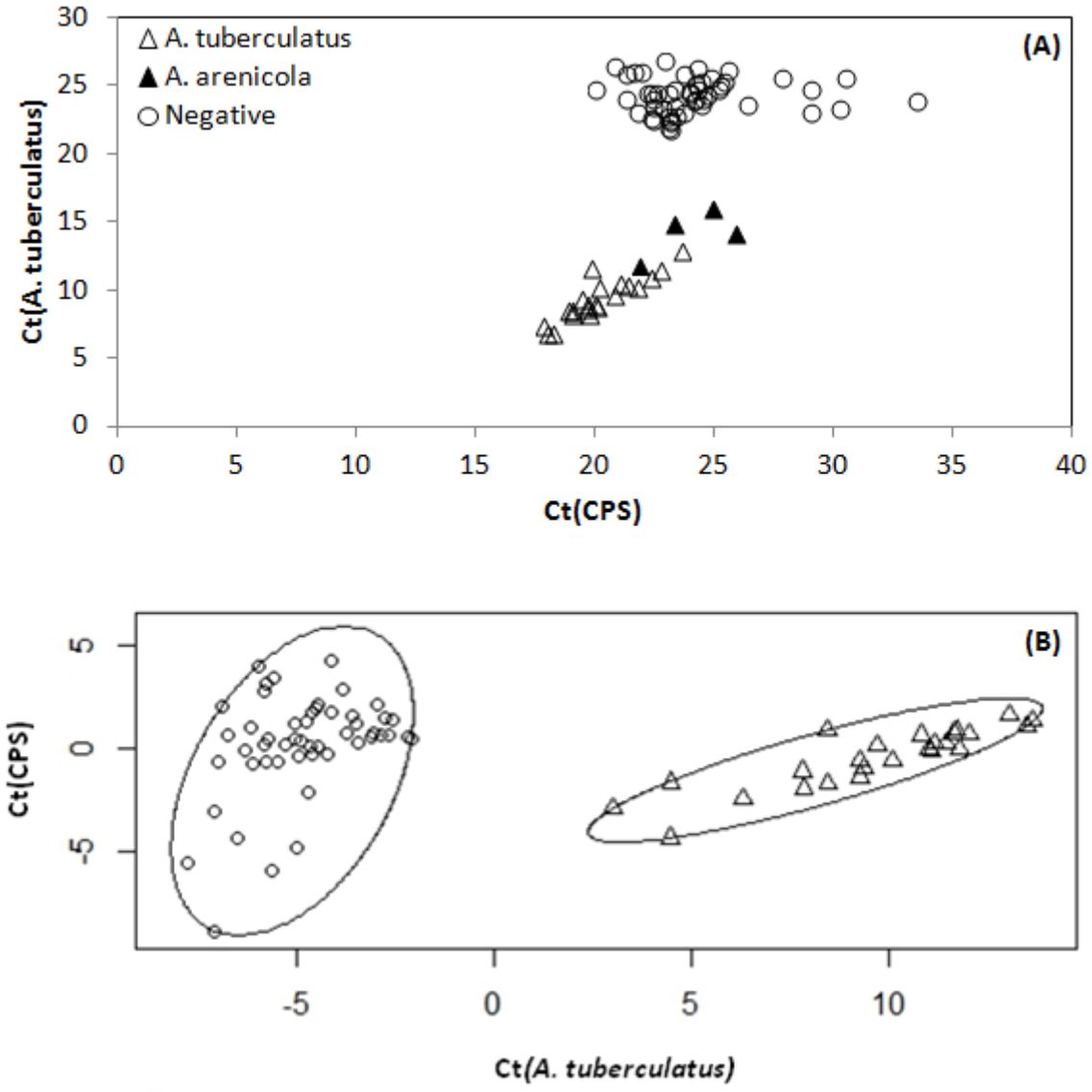

Figure 2.3. Quantitative polymerase chain reaction cycle threshold (Ct) values of *A. tuberculatus* and reference gene CPS across *Amaranthus* diversity panel. (A) Ct values by expected group. *A. arenicola* was not represented in primer design. Negative group is all remaining *Amaranthus* spp. (B) K-mer cluster analysis of Ct values, K = 2. The number of data points underestimates the number of samples because some data points overlap. *A. tuberculatus* and *A. arenicola* accessions are observed within the same group.

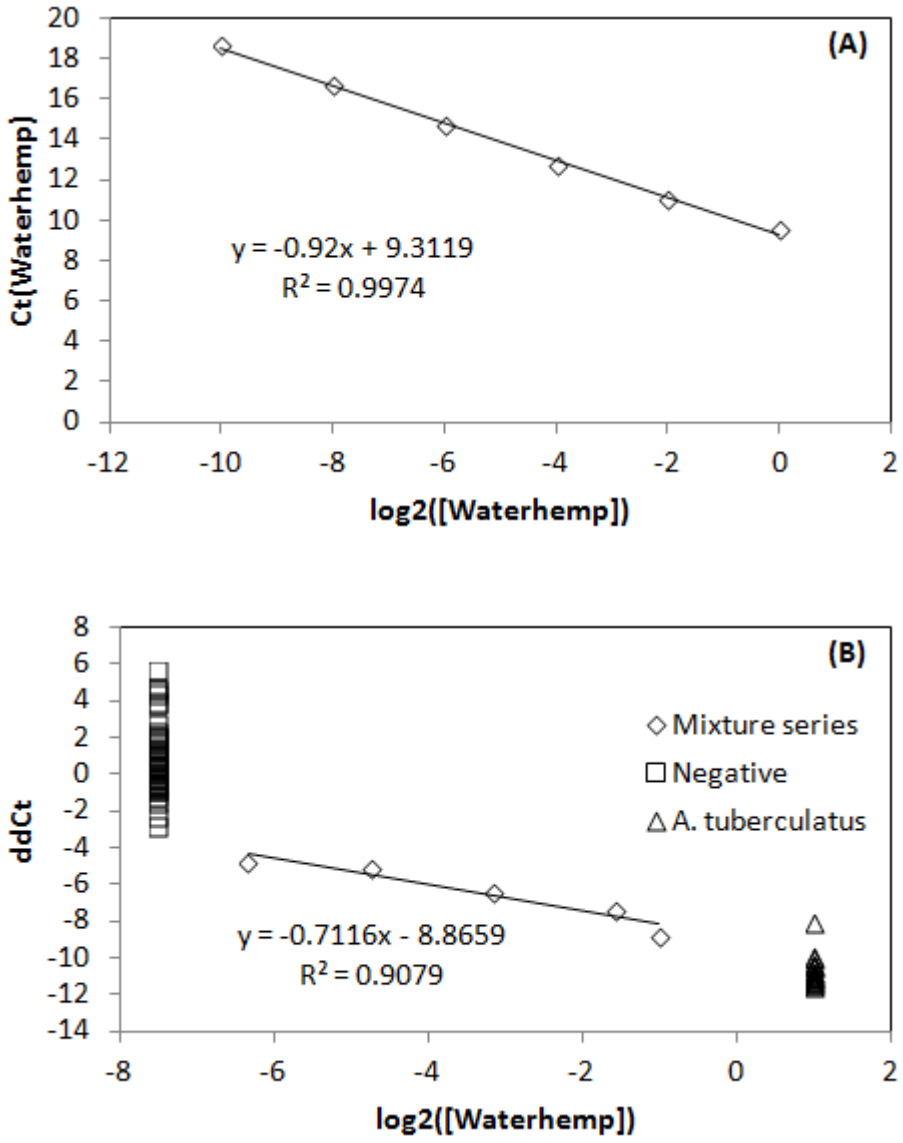

Figure 2.4. Quantitative polymerase chain reaction of simulated population samples. (A) dilution series (with water) of DNA from an *A. tuberculatus* accession. (B) Mixture series of *A. tuberculatus* and *A. palmeri* DNA as simulated population samples. *A. tuberculatus* leaf DNA provides a positive control. Other accessions of the diversity panel provide reference for a no-template control. The number of data points underestimates the number of samples due to overlap.

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CHAPTER 3: A QUANTITATIVE ASSAY FOR *AMARANTHUS PALMERI* IDENTIFICATION[2]

ABSTRACT

Amaranthus palmeri recently has been brought into the Midwestern USA as a contaminant in Conservation Reserve Program seeding mixes. Rapid species screening is required to mitigate the risk of continued species movement. Markers were developed for *A. palmeri*-specific nucleotide polymorphisms in the internal transcribed spacer of the ribosomal coding region. A quantitative polymerase chain reaction (qPCR) assay successfully identified *A. palmeri* from single-plant samples, simulated mixed-plant samples and seed mixtures. A qPCR assay for distinguishing *A. palmeri* from 12 other *Amaranthus* spp. was developed and validated. The assay can consistently detect a single *A. palmeri* seed when present in a pool of 100 total *Amaranthus* spp. seeds.

INTRODUCTION

The establishment of conservation acres is known to result in the movement of mixed seeds between geographically isolated areas. In Iowa, introductions of *Amaranthus palmeri* have been confirmed in 48 counties in 2016 (Hartzler and Anderson 2016). Similar introductions of *A. palmeri* have occurred in Illinois and likely in several other Midwestern US states (Hager AG, personal communication). These introductions have been observed in fields seeded to Conservation Reserve Program (CRP) hectares as a result of contaminated out-of-state seed (Hartzler and Anderson 2016). Currently, *A. palmeri* is legally considered a prohibited noxious weed in the states of Delaware, Minnesota and Ohio (Henseigh and Pokorny 2017) (and being

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considered for similar classification in other states). As a prohibited noxious weed, *A. palmeri* cannot be present as a contaminant in seed sold in these states. There are higher tolerances for seeds of other *Amaranthus* species in commercial seed and, consequently, there is a need to reliably distinguish *A. palmeri* seeds from those of other *Amaranthus* species.

Several molecular assays have been developed for the differentiation of *Amaranthus* spp. A restriction fragment length polymorphism assay was developed utilizing the internal transcribed spacer (ITS) of the ribosomal coding region (Wetzel et al 1999). Additionally, a polymerase chain reaction (PCR)-based assay was developed utilizing the intron 1 sequence of 5-enolpyruvylshikimate-3-phosphate synthase gene (Wright et al 2016). These assays are limited to identification of single-plant samples.

The goal of the study presented here was to develop a quantitative molecular assay for *Amaranthus* spp. identification. We present a quantitative PCR (qPCR)-based assay for identification of *A. palmeri* from 12 other *Amaranthus* spp., and demonstrate its applicability to mixed-sample screening.

MATERIALS AND METHODS

Species-specific Single Nucleotide Polymorphism (SNP) Identification

Ribosomal RNA sequence from *A. albus* (Genbank accession number JF975853) was queried against the NCBI nucleotide database using blastn with default parameters, resulting in 90 hits within the *Amaranthus* genus (Taxonomy ID: 3564). The sequences of species represented three or more times in this list were extracted and aligned using MAFFT and SNPs specific to *A. palmeri* were manually identified.

Plant Accessions

At least ten accessions each of *A. blitoides, A. hybridus, A. palmeri, A. powellii, A. retroflexus* and *A. tuberculatus* were obtained from University of Illinois in-house collections. Five accessions each of *A. albus, A. arenicola, A. blitum, A. hypochondriacus, A. spinosus, A. tricolor* and *A. viridis* were obtained from the Germplasm Resources Information Network (GRIN). Seeds were grown under greenhouse conditions as described previously (Patzoldt et al 2006). The species identification of each accession was confirmed through morphological characterization and the previously developed ITS assay (Wetzel et al 1999).

DNA Extraction

Biological triplicates of young leaf tissue were collected from one plant of each accession. Leaf DNA extraction followed a CTAB procedure (Doyle and Doyle 1990). DNA was quantified with Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 100 ng/μL. Seed DNA extractions were performed on six replicate pools of 100 seeds, each comprised of *A. tuberculatus* seeds plus one or two *A. palmeri* seeds. Seed DNA extraction followed the FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA, USA) with the following modifications: seeds were incubated overnight at room temperature in cell lysis solution, and the homogenization step was conducted twice. Seed DNA extractions were repeated in time, yielding a total of 12 replications.

qPCR

Primers for *A. palmeri* identification (forward, 5′-GCGAACATGTTTATCATACCTGG-3′; and reverse, 5′-CTCAATACTGGGTGCATCCAC-3′) were designed using *A. palmeri*-specific SNPs. Each qPCR reaction consisted of 5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 1 μL of forward primer, 1 μL of reverse primer and 1 μL pf

template DNA. A previously identified qPCR control gene *CPS* was included as a single copy control (Ma et al 2013). The qPCR thermoprofile was as follows: 95 °C for 5 min; 35 cycles of 59 °C for 1 min and 95 °C for 15 s; immediately followed by a melt curve analysis for quality control of positive samples (Ma et al 2013). Technical duplicates were used to minimize error. An arbitrarily chosen *A. palmeri* accession was used for a six-step, 1:4 dilution series of the sample with water. A 1:1 mixture of the same *A. palmeri* accession and an arbitrarily chosen *A. tuberculatus* accession was used to simulate mixed samples, which was used for a six-step, 1:3 dilution series with *A. tuberculatus* DNA. A subset of accessions was arbitrarily chosen for qPCR analysis with no fewer than three accessions present for each species. For analysis of seed samples, qPCR was performed using 1 µL of undiluted DNA obtained from each of the 100-seed extractions; qPCRs using DNA from *A. tuberculatus* and *A. palmeri* were included as negative and positive controls, respectively.

Statistical Analysis

A k-mer cluster analysis ('cluster' R package) was used to differentiate accessions (k = 3). The ddCt method was used to analyze mixture series (Schmittgen and Livak 2008; Taylor et al 2010).

RESULTS

Amaranthus palmeri qPCR Identification

qPCR and k-mer cluster analysis distinguished *A. palmeri* accessions from all other accessions (Figure 3.1). *Amaranthus tuberculatus* accessions clustered more closely to *A. palmeri* than did the other species. *Amaranthus arenicola* accessions clustered with negative samples (all other accessions except *A. palmeri* and *A. tuberculatus*), despite not being used for

primer design. Based on the lack of overlap between the negative and *A. palmeri* clusters (Figure 3.1), no false positives are expected to be observed using this assay with the species investigated.

Quantification of Mixed Samples

A dilution series of an arbitrarily chosen *A. palmeri* accession revealed high sensitivity for the *A. palmeri*-specific marker (Figure 3.2A). A simulated seed mixture series resulted in a strong linear relationship between predicted and actual concentrations of *A. palmeri* DNA when diluted with *A. tuberculatus* DNA. At 1:81 dilution of *A. palmeri* DNA with *A. tuberculatus* DNA (the highest dilution tested), the predicted concentration was differentiable from all undiluted *A. tuberculatus* accessions (Figure 3.2B). *Amaranthus tuberculatus* was included as a 'worst case' negative *Amaranthus* spp. because it clustered closest to *A. palmeri* (Figure 3.1). When the assay was conducted on seed mixtures, *A. palmeri*-containing mixtures were consistently differentiated from the negative control (*A. tuberculatus*) even when there was only one *A. palmeri* seed in the 100-seed pools (Figure 3.2C).

DISCUSSION AND CONCLUSION

Movement of seed through the CRP has been implicated in the recent biological invasion of *A. palmeri* into the Midwest USA (Hartzler and Anderson 2016). Screening of seed mixtures would mitigate risks of similar invasions occurring in the future. Here, we have created a qPCR assay for the identification of *A. palmeri* for use in mixed seed samples.

The *A. palmeri* marker successfully differentiated *A. palmeri* from all other *Amaranthus* spp. investigated in this study. Additionally, *A. tuberculatus* accessions cluster separately from all other *Amaranthus* spp. with the *A. palmeri* marker; however, slight overlap between negative and *A. tuberculatus* eclipses is observed. The cause for the amplification of *A. tuberculatus* with

this marker is currently unknown. On a single plant basis, our assay can differentiate *A. palmeri* from all other tested *Amaranthus* spp.

Quantification of species presence in a mixed plant sample allows improved efficiency for contaminated seed screening. Previously, plant grow-outs followed by inspection of floral morphology was used to identify *A. palmeri* contaminants in CRP seedlots (Hartzler and Anderson 2016). The assay developed in this study allows the differentiation and quantification of *A. palmeri* in mixed samples. Based on the *A. palmeri* dilution series, assay sensitivity could reach 1:256 *A. palmeri* to *A. tuberculatus*. In practice, we were able to consistently detect a single *A. palmeri* contaminant in pools of 100 seeds. Based on the separation of the ddCt values between the 1:100 mixtures and *A. tuberculatus* controls, we believe the assay could be sensitive at lower contamination levels. The current limitation in assay sensitivity likely is the difficulty in extracting DNA from every seed in large seed pools. While the limit of detection of *A. palmeri* in mixed samples has yet to be optimized, this study describes the first molecular assay for detection and quantification of *A. palmeri* in mixed samples.

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FIGURES

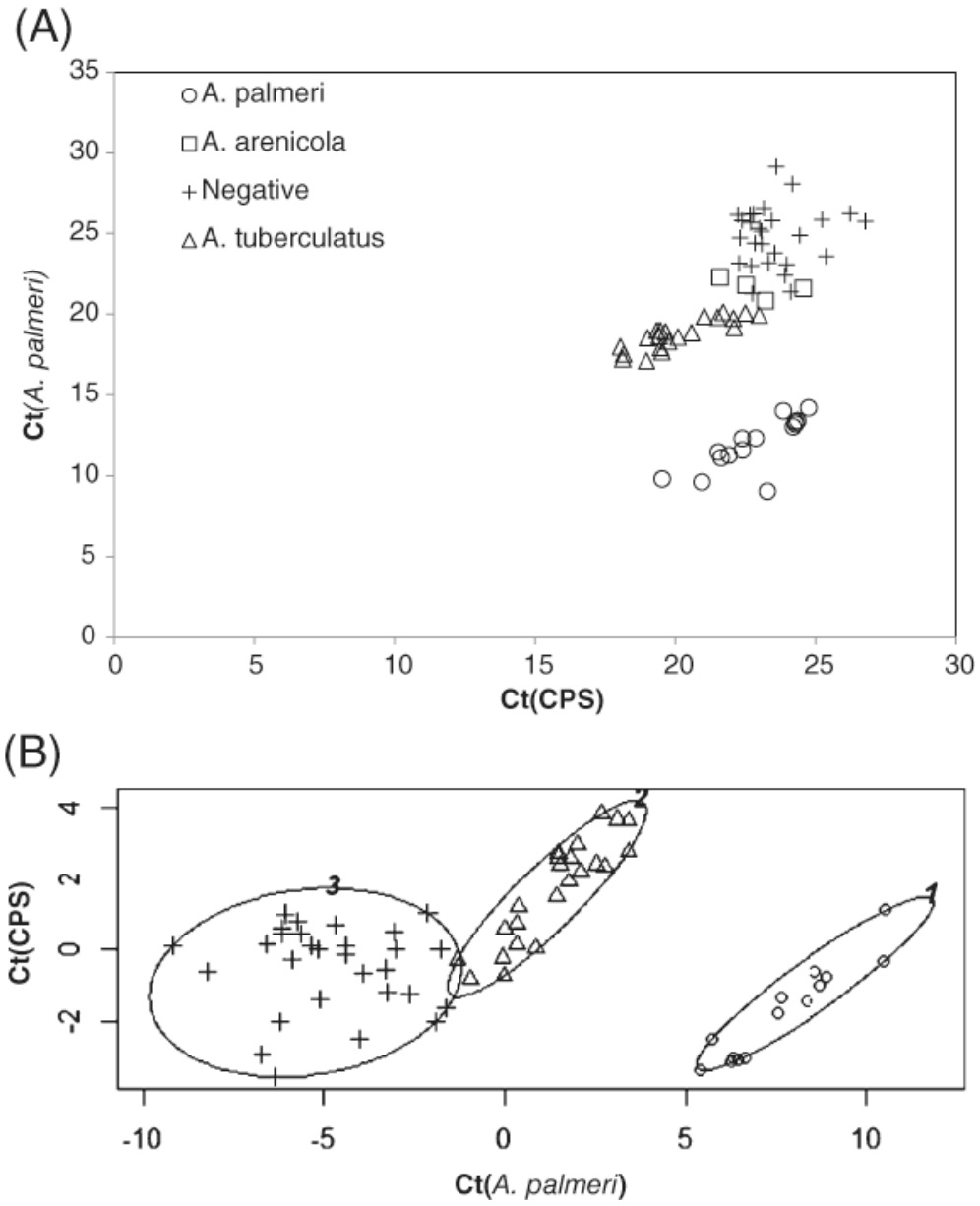

Figure 3.1. Quantitative polymerase chain reaction cycle threshold (Ct) values of *A. palmeri* and reference gene CPS across *Amaranthus* diversity panel. (A) Ct values by expected group. *Amaranthus arenicola* was not represented in primer design. Negative group is all remaining *Amaranthus* spp. (B) k-mer cluster analysis of Ct values, k = 3. The number of data points underestimates the number of samples because some data points overlap.

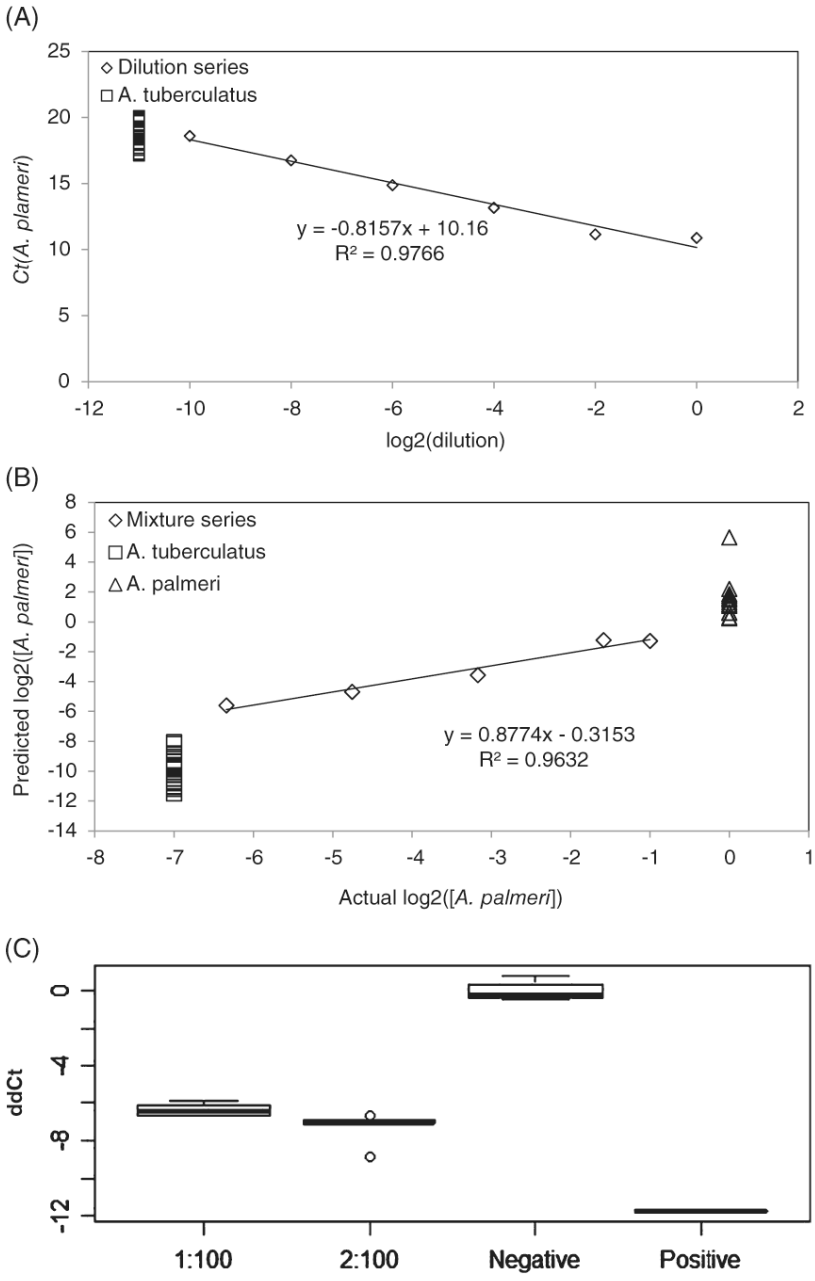

Figure 3.2. Quantitative polymerase chain reaction of simulated and actual mixed seed samples. (A) Dilution series (with water) of DNA from an *A. palmeri* accession. (B) Predicted *A. palmeri* concentration of simulated mixed seed samples (obtained by mixing *A. palmeri* and *A. tuberculatus* DNA). (C) Box plots (n = 12) for seed mixtures (100-seed pools) containing *A. tuberculatus* seeds plus one (1:100) or two (2:100) *A. palmeri* seeds. *Amaranthus tuberculatus* (Negative) and *A. palmeri* DNA (Positive) served as controls.

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CHAPTER 4: HERBICIDE-RESISTANCE SURVEILLANCE IN OHIO SOYBEAN FIELDS

ABSTRACT

Herbicide resistance within key driver weeds, such as *Amaranthus tuberculatus*, constrains available management options for producers. Routine surveillance for herbicide resistance allows the buildup and movement of resistant populations to be observed over time. Furthermore, the identification and quantification of resistance mechanisms on a population level can aid in management. Populations of *Amaranthus* spp. were collected from 51 fields in Ohio during the 2016 growing season. Twenty-four populations were screened for resistance to the herbicides lactofen, atrazine, and glyphosate. Phenotypically resistant plants were further investigated for known resistance mechanisms. Resistance to lactofen was sparsely observed throughout the surveyed populations, with only two of 22 populations possessing high levels of resistance. The ΔG210 resistance mechanism was observed in the majority of cases, however some resistant plants lacked all known resistance mechanisms. Resistance to atrazine was observed at a low frequency throughout most populations, though several populations had high incidence of resistance. No known resistance mechanisms were observed within the screened populations. Resistance to glyphosate was observed at high frequencies throughout all surveyed populations. Gene amplification was the predominant resistance mechanism, though the P106S mutation was observed in a number of cases. Multiple plants with both resistance mechanisms were identified. In this study, molecular screening underestimated the phenotypically observed resistance in most cases. Continued mechanism discovery and marker creation is required to better approximate phenotyping.

INTRODUCTION

Herbicide-resistant weeds emerged as a limiting factor in agricultural production in the 1970s (Heap 2014). Currently, herbicide resistance to several sites of action (SoA), such as 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), psbA of photosystem II (PSII), and protoporphyrinogen oxidase (PPO), are increasing in species frequency (Heap 2018) and distribution (Chatham et al 2014; Schultz et al 2014; Vieira et al 2018). Perhaps as a result of decreased control options, herbicide-resistance traits often are highly concentrated on driver weed species, such as *Amaranthus tuberculatus*, *Amaranthus palmeri*, and *Lolium rigidum* (Heap 2018).

Amaranthus tuberculatus is considered one of the most troublesome weeds throughout much of the Midwestern United States (Legleiter and Bradley 2008; Tranel et al 2010; McMullan and Green 2011; Vieira et al 2018). It is an annual, dioecious weed with an extended germination period and prolific seed production (Costea et al 2004). Currently, herbicide resistance to six SoA have been documented in *A. tuberculatus* (Heap 2018). Fortunately, herbicide resistance to some, but not all, SoA are highly localized to specific fields. However, resistance to other SoA such as EPSPS are widespread across multiple states, as observed through state-wide surveys (Chatham et al 2014; Scultz et al 2014; Vieira et al 2018). Within each survey, pockets of susceptible *A. tuberculatus* populations were observed. Maintaining separation between resistant and sensitive fields can allow for more control options on a field-by-field basis. Investigating existing pockets of susceptibility can inform management decisions to limit the spread of resistance.

The identification of molecular mechanisms endowing resistance to herbicides is a necessary component of field surveillance initiatives. Certain mechanisms are known to cause

resistance to some, but not all, classes of herbicides that target the same SoA. For instance, the Y222F mutation of the *psbA* enzyme endowing resistance to atrazine, a PSII-inhibitor, results in super-sensitivity to diuron, another PSII-inhibitor of a different family (Oettmeier 1999). Additionally, the mechanism of resistance can influence how resistance spreads. Target site resistance to atrazine is maternally inherited (Oettmeier 1999), while non-target site resistances are often inherited in a Mendelian manner (Huffman et al 2015). Nuclear inheritance allows for the reliable spread of traits through pollen. Finally, understanding the molecular mechanism endowing herbicide resistance allows the creation of molecular markers specific to the mechanism. The discovery of glyphosate resistance mediated through gene amplification of *EPSPS* enabled the development of a quantitative polymerase chain reaction (qPCR) assay to quantify the degree of amplification, enabling routine screening for this mechanism (Gaines et al 2009). Should a mechanism, or series of mechanisms, of resistance be determined to be highly prevalent within a geographic region, screening for simply the mechanism in the absence of phenotyping may approximate total observable resistance.

Currently, a variety of molecular diagnostics are available to diagnose resistance to EPSPS-inhibitors, PSII-inhibitors, and PPO-inhibitors. A gene amplification qPCR assay has been routinely used to identify glyphosate resistance (Gaines et al 2009). Additionally, a derived cleaved amplified polymorphic sequences (dCAPS) assay is available for the resistance-endowing P106S mutation of EPSPS (Chatham et al 2015). Limited molecular markers are available for nontarget-site resistance to atrazine, however an RFLP assay targeting the psbA gene is available (Foes et al 1998). Recently, the overexpression of the glutathione *S*-transferase *AtuGSTF2* was linked to metabolic atrazine resistance (Evans et al 2017), however assaying for this requires RNA which is impractical for surveillance. Several molecular markers have become

available for PPO-inhibitor resistance. A TaqMan assay specific to the ΔG210 of PPO, which endows resistance to PPO-inhibitors was developed (Lee et al 2008). Additionally, substitutions at R128 may also endow resistance to PPO inhibitors (Giacomini et al 2017). While dCAPS assays for the R128 substitutions exist for *Amaranthus palmeri* (Giacomini et al 2017), no such assay exists specific to *A. tuberculatus*.

Recently, a state-wide herbicide-resistance surveillance program was established in Ohio. Resistance surveillance allows for the identification of multiple resistances existing within a given field, impacting available control options. Additionally, surveillance can identify pockets of susceptibility, allowing isolation and localized maintenance of available control options. This work herein describes a state-wide survey of PSII-inhibitor, PPO-inhibitor, and EPSPS-inhibitor resistance in *A. tuberculatus*. Molecular mechanisms, in addition to resistant phenotypes, are quantified at a population level.

MATERIALS AND METHODS

Seed Collection

Amaranthus spp. seed was collected during a late-season survey of weed infestations in soybean fields. Fifty-one counties were surveyed within Ohio. Sampling was restricted to fields with infestation patterns indicative of herbicide resistance issues. Composite samples of several plants were taken to reflect the population present within the sampled field. Species of samples were determined, and *A. tuberculatus* samples were used for herbicide screening

Plant Growth Conditions

Seeds from 22 populations and the known sensitive control accession *WUS* (We et al 2018) were surface sterilized with 50% fresh bleach, washed with deionized water and kept at

4ºC for five weeks before sown. Plants were germinated in Petri dishes at 35ºC on 12-hour days. Seedlings were transplanted into individual cone-tainers (Stuewe and Sons Inc, OR, USA) filled with 3:1:1:1 Sunshine LC1: soil:peat:torpedo sand. Plants were kept in the greenhouse at 12-hour days, 28-30ºC during the day and 25-27ºC at night. Plants were watered twice daily with mist irrigation.

Herbicide Application

Up to 20 plants at the 4-6 leaf stage from each population were selected for uniformity. Herbicide applications with glyphosate (Roundup Weathermax, Monsanto Company) at 1x field rate (840 g ae ha^{-1}) and 1.5x field rate (1260 g ae ha^{-1}), atrazine (Atrazine 90DF, Winfield Solutions) at 1x field rate (1284 g ai ha^{-1}), and lactofen (Cobra, Valent USA Corporation) at 0.03x field rate (5.48 g ai ha^{-1}). Additives were included based on label requirements. Plants were sprayed using a moving-nozzle cabinet spray chamber using a 80015 even flat fan nozzle (TeeJet Technologies) applied 46 cm above the plant canopy. Spray volume was calibrated for 187 L ha^{-1}. Three weeks after glyphosate treatment, and two weeks after lactofen or atrazine treatment, each plant was visually rated for survivorship. Sensitive and resistant plants were delimited by the presence of new green tissue appearing after herbicide application. A total of 22 populations were screened for resistance to lactofen, 13 populations were screened for resistance to atrazine, 16 populations were screened for resistance to glyphosate at a 1x field rate, and 15 populations were screened for resistance to glyphosate at a 1.5x field rate (Table 4.1).

DNA Extraction

Single leaf samples were taken from all surviving plants and several untreated *WUS* plants. Hexadecyltrimethylammonium bromide (CTAB) DNA extraction procedure (Doyle and Doyle 1990) was conducted on all leaf samples. Samples were assessed for quality and DNA

quantity using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). All DNA samples were diluted to 10ng μL^{-1} for molecular diagnostics.

EPSPS Gene Amplification

qPCR was conducted on survivors of 1x and 1.5x glyphosate applications to determine EPSPS gene copy number. A single copy control gene carbamoylphosphate synthase (*CPS*) was used as a reference gene (Ma et al 2013). qPCR protocol was described by Ma et al (2013). The ΔΔCt method was used to determine gene amplification (Ma et al 2013). A ΔΔCt threshold of -1.14, three standard deviations from the mean value of susceptible control samples, was used to delimit samples with the gene amplification mechanism.

EPSPS P106S Substitution

A dCAPS assay was conducted on all survivors of the 1x glyphosate applications to determine presence of the Pro106Ser substitution (Chatham et al 2015). PCR and digestion were performed as described previously (Chatham et al 2015), and bands were visualized on 2% agarose gel stained with GreenGlo Safe DNA Dye (Denville Scientific Inc).

PPO ΔG210 Deletion Assay

A TaqMan qPCR assay was conducted on all 0.03x lactofen survivors. The assay was conducted as described by Wuerffel et al (2015).

PPO R128 Substitutions

A 500bp region of the PPO gene from all 0.03x lactofen survivors was sequenced as described by Giacomini et al (2017) with the following modifications. Initial PCR was conducted with primers southernF (TCCATTACCCACCTTTCACC) and southernR (AGCGGGATTTGAAGGTAGTAG). A second PCR was conducted using the same forward primer and the reverse primer R98M_XhoII (AGCGGGATTTGAAGGTAGTAG).

Amplification products were confirmed on 1% agarose gel stained with GreenGlo Safe DNA Dye, and PCR products were purified with the EZNA Gel Extraction Kit (OMEGA Bio-tek Inc). Sequencing was conducted on purified PCR products.

Ser264Gly Substitution

A PCR-RFLP assay was conducted on all 1x atrazine survivors, as described by Schultz et al (2015).

Distribution Maps

The web program PhyloGeoVis (phyolgeoviz.org) was used to generate kml files based on sample location data, herbicide resistance screening, and mechanism identification. A Google map of Ohio was used as an underlay for the generated kml file.

RESULTS AND DISCUSSION

A mixture of *A. tuberculatus*, *A. retroflexus*, and *A. palmeri* was observed in the statewide samples (Figure 4.1). *Amaranthus tuberculatus* samples were primarily clustered to the west, while *A. retroflexus* was observed throughout the state. *Amaranthus palmeri* was infrequently observed throughout the state. In general, samples representing the northwest corner, and east-southeast were absent. While the East-Southeast has limited soybean production (USDA-NASS 2016), the northwest corner observes production similar to surveyed regions. Of the 51 populations collected, 29 were *A. tuberculatus*. Of these populations, 24 had sufficient seed availability and germinability for phenotypic screening with at least one herbicide.

Herbicide resistance to glyphosate was high throughout the region (Figure 4.2). At 1x field rate, limited survivorship was observed within the sensitive *WUS* control, causing an additional test at 1.5x field rate to be conducted on a subset of populations. Therefore, the group

of survivors with no known mechanism of resistance for a given population, termed group ‘unknown’, may be composed of both true resistant plants and escaped sensitive plants. When comparing populations that were screened for resistance at both 1x and 1.5x field rates, survivorship is similar in most cases (Table 4.2), though prevalence of the gene amplification mechanism varied (Table 4.3). Reduced sensitivity to glyphosate has been reported under similar circumstances in Nebraska (Vieira et al 2018), and the presence of a third mechanism of resistance has been proposed by Chatham et al (2015).

In general terms, gene amplification appears to be the dominant form of glyphosate resistance present in Ohio, present within 34% of screened plants at both 1x and 1.5x field rate. Of resistant plants, 49% possess gene amplification. Gene amplification has been reported as the dominant form of resistance in Missouri and Illinois (Chatham et al 2015; Vieira et al 2018). Additionally, the prevalence of the P106S substitution was low (5%) relative to gene amplification mechanism, similar to observations in Missouri and Illinois (Chatham et al 2015; Vieira et al 2018). The prevalence of the P106S mechanism was not investigated within the 1.5x field rate survivors as allele frequency observed in the 1x field rate survivors was low. When including the ‘unknown’ group from the glyphosate 1x field rate application, approximately 74% of plants screened produced a resistant phenotype to glyphosate. The high rate of resistance could be a result of glyphosate being applied to the sample location early in season, selecting against glyphosate sensitive *A. tuberculatus*. When combining both screening rates, close to 50% of resistant plants fell into the ‘unknown’ category. Multiple instances of ‘double’ resistant plants were observed, where both gene amplification and the P106S mutation were observed. Should both resistance mechanisms be present within a given population, recombination between them is expected to occur at some frequency, giving rise to these ‘double’ mutant plants. We

cannot comment on if P106S exists within duplicated copies of the EPSPS gene. Perhaps a CASFISH approach (Deng et al 2015), where a dCRISPR-dCas9 probe specific to the P106S allele is created and applied to selected plants would provide insight into the placement of the mutation within the genome. Previously, Chatham et al (2015) proposed that a third mechanism of resistance may be present within Illinois *A. tuberculatus* populations. The prevalence of resistant plants without a known resistance mechanism in Ohio not only supports this observation, but provides evidence this 'third mechanism' may not be specific to Illinois. From both Ohio and Illinois state-wide surveys, there is growing evidence for the existence of at least one unknown mechanism of glyphosate resistance in *A. tuberculatus* (Nandula et al 2013).

Herbicide resistance to lactofen was observed in low frequencies throughout much of the surveyed populations (Figure 4.3). In fact, 14 of the 22 screened populations were observed to have no survivors of the lactofen treatment. The majority of observed resistance was concentrated within few populations, indicating resistance to PPO-inhibitors may be highly localized. The identification of fields with and without regionally rare herbicide resistances allows for isolation and maintenance of susceptible alleles throughout the region. Two populations had a relatively high level of resistance to lactofen. In Population 19, the ΔG210 deletion was present in 35% of screened plants. In Population 33, the ΔG210 deletion was present in 45% of screened plants. No instances of R128 substitutions were observed within the screened populations. Several instances of surviving plants without a known resistance mechanism were identified. In the case of Population 14 (one survivor) and Population 60 (three survivors), survivors of which had no known mutations, herbicide screening was replicated, resulting in 40 plants screened per population. Within the second screening, no resistant plants were observed. It remains unclear if the surviving plants without known resistance mechanisms

are truly resistant or were simply escapes. The populations remain under continued screening to obtain additional resistant individuals for further analysis.

Herbicide resistance to atrazine was observed in low frequencies throughout much of the surveyed populations (Figure 4.4). Interestingly, only Population 14 had no resistant plants, however this could be an artifact of lowered sample number. Several populations, such as Population 1 (95% survivorship) and Population 19 (46% survivorship), had high survivorship. The presence of atrazine resistance within nearly all tested populations indicates that atrazine could be considered a 'last resort' herbicide. While an atrazine application may be able to control *A. tuberculatus* at a reasonable level in most fields, the presence of resistance at measurable levels would indicate repeated application could swiftly lead to herbicide failure (Jasieniuk et al 1996). The sole known characterized target-site resistance mechanism to atrazine in *A. tuberculatus*, the G264S substitution, was not observed in any resistant plants. Currently, no reliable genomic based assays exist for *GST*-mediated resistance to atrazine. Existing assays rely on RNA (Evans et al 2017), which is impractical for routine surveillance.

As new resistance alleles are characterized, the ability to quantify resistance through mechanism screening will begin to approach phenotyping. However, as observed in both glyphosate and lactofen survivors, substantial gaps may exist in our knowledge of resistance mechanisms. Surveillance provides a unique opportunity to not only quantify resistance on a field basis over a large region, but also identify populations that harbor new resistance alleles for characterization. Continued screening of targeted populations will not only increase our understanding of how herbicide resistance changes over years, but can lead to the identification of populations where new resistance mechanisms have been enriched to the point where characterization is required.

All populations were collected from infested soybean fields near the end of the season. Glyphosate is a commonly applied herbicide in soybean production. While the herbicide use history of the surveyed fields is unknown, in-season application of glyphosate would enrich the current population for glyphosate-resistant plants. Should a subset of the field be left untreated and subsequently sampled, the proportion of any given population possessing glyphosate resistance may decrease.

A further consideration is how fields were selected for sampling. Infested fields were specifically targeted for population collection. If a population had very low or no resistance to the herbicide program implemented on a given field, the field would not be considered for sampling. The sampling procedure enriched the population set for resistance to the dominant herbicide programs within the region, therefore high levels of resistance to at least one herbicide is expected. Should a random sampling approach be taken across the state, the frequency of resistance observed in this project may decrease.

In summary, this study suggests that glyphosate resistance is widespread within Ohio soybean fields. The major mechanism of glyphosate resistance appears to be gene amplification, however unknown mechanisms of resistance likely exist within the surveyed populations. Resistance to PPO-inhibiting herbicides appears infrequent but concentrated in few populations, providing an opportunity for use on a field-to-field basis. Resistance to atrazine was observed at low frequencies across nearly all screened populations, indicating that the herbicide could be viewed as a ‘last resort’ herbicide, and may fail in the near future.

TABLES AND FIGURES

Table 4.1. Numbers of plants from Ohio *A. tuberculatus* populations sprayed with herbicides lactofen (0.03x field rate), atrazine (1x field rate), and glyphosate (1x and 1.5x field rate).

Population Number	Latitude	Longitude	Lactofen (0.03x)	Atrazine (1x)	Glyphosate (1x)	Glyphosate (1.5x)
1	40.9594	-81.001	20	20	20	20
2	40.96	-81.003	20	0	0	20
3	40.9164	-80.926	20	0	0	0
4	40.9145	-80.713	20	0	0	0
10	39.8305	-83.772	20	0	20	0
11	39.8305	-83.777	20	20	0	0
13	40.2544	-84.694	0	0	0	20
14	40.3051	-84.683	40	15	20	0
16	40.3729	-84.672	20	0	20	20
17	40.4238	-84.677	20	0	20	0
18	40.4915	-84.689	20	15	20	0
19	40.6101	-84.689	20	15	20	0
20	40.6778	-84.667	20	0	0	0
30	40.8305	-81.887	20	15	20	20
31	40.8305	-81.887	20	15	20	20
33	40.8305	-81.887	20	15	20	20
35	40.678	-83.862	20	20	20	20
36	40.8644	-83.563	20	15	20	20
55	38.9659	-84.087	20	0	0	20
60	40.0742	-84.707	40	15	20	20
62	40.3517	-84.627	20	15	20	20
67	40.6154	-83.915	20	15	20	20
69	40.6046	-84.299	20	0	20	20
70	40.4351	-84.415	0	0	0	20

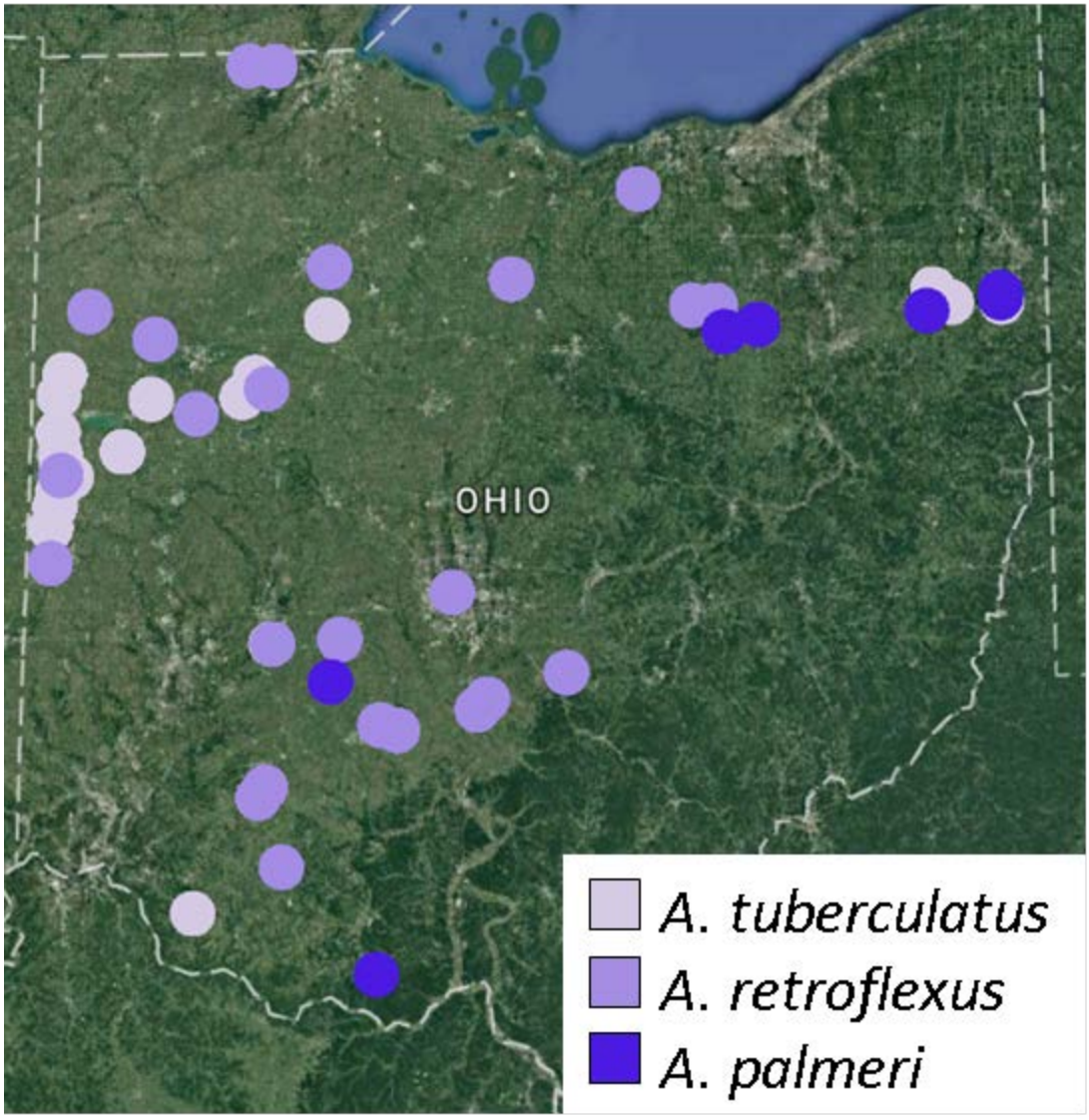

Figure 4.1. Species distribution of *Amaranthus* spp. through the Ohio state-wide survey.

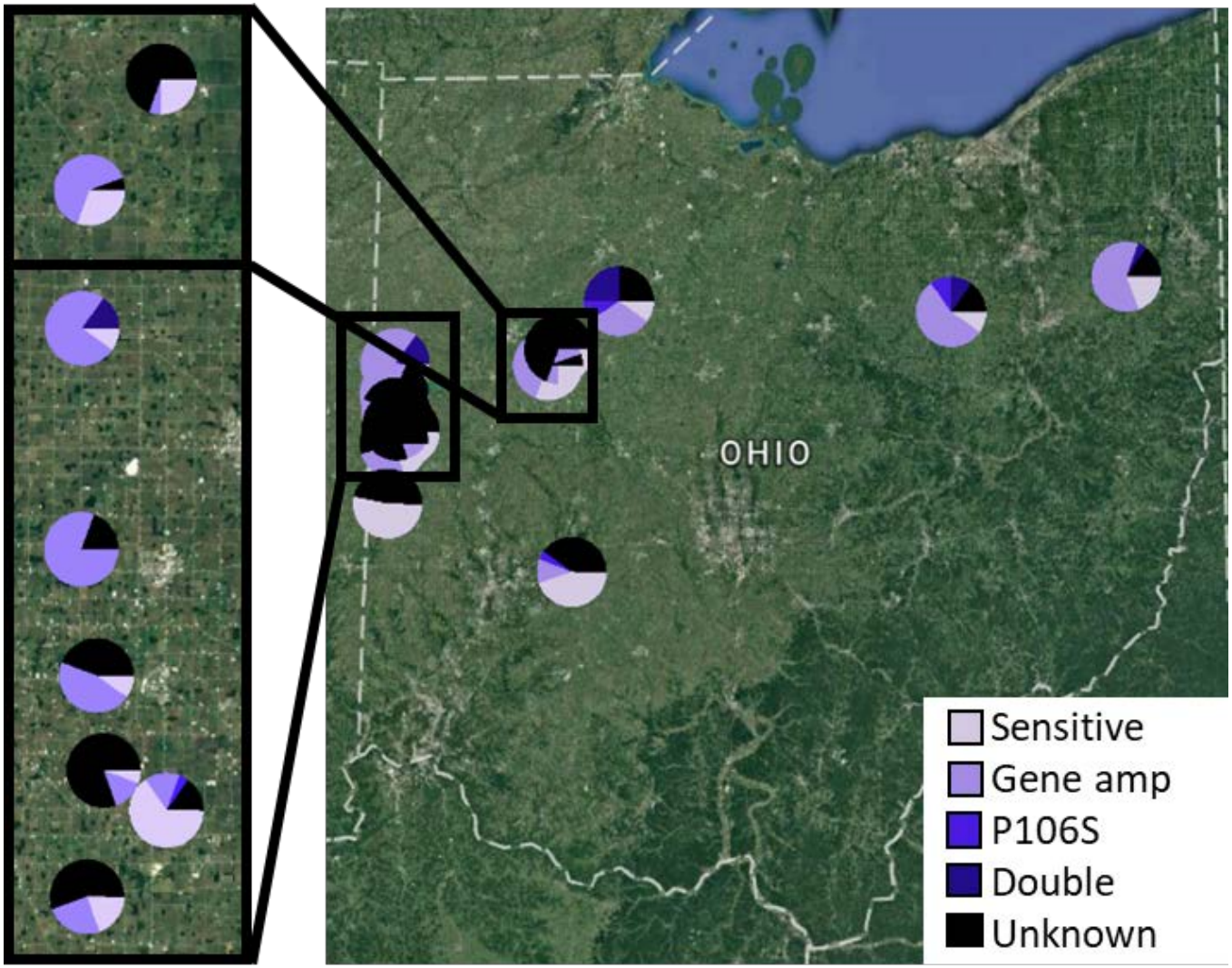

Figure 4.2. Distribution of glyphosate-resistance mechanisms within *A. tuberculatus* populations collected during the Ohio state-wide survey. Populations were screened at 1x field rate.

Table 4.2. Survivorship of Ohio population samples to lactofen, atrazine, and glyphosate applications

Population Number	Lactofen survivorship	Atrazine survivorship	Glyphosate (1x) survivorship	Glyphosate (1.5x) survivorship[a]
1	0/20	19/20	16/20	4/20
2	0/20	NA	NA	18/20*
3	0/20	NA	NA	NA
4	0/20	NA	NA	NA
10	1/20	NA	11/20	NA
11	0/20	2/20	NA	NA
13	NA	NA	NA	20/20*
14	1/40	0/15	16/20	NA
16	0/20	NA	19/20	14/20*
17	0/20	NA	18/20	NA
18	0/20	1/15	20/20	NA
19	8/20	7/15	18/20	NA
20	0/20	NA	NA	NA
30	0/20	3/15	12/20	18/20
31	0/20	5/15	18/20	20/20
33	12/20	4/15	6/20	10/20
35	0/20	3/20	15/20	10/20*
36	0/20	1/15	18/20	17/20
55	0/20	NA	NA	10/20
60	3/40	0/15	14/20	14/20*
62	1/20	3/15	7/20	17/20
67	1/20	1/15	14/20	12/20
69	3/20	NA	10/20	4/20
70	NA	NA	NA	15/20*

[a] only a subset of glyphosate 1.5x survivors were selected for gene amplification mechanism evaluation, denoted with *. Subset composed all populations not screened at 1x field rate and a random selection of overlap between the two doses.

Table 4.3. Gene amplification frequency within Ohio populations screened at 1x and 1.5x field rate glyphosate

Population Number	Glyphosate (1x) survivorship	Glyphosate (1.5x) survivorship[a]	Gene amplification (1x)	Gene amplification (1.5x)
16	19/20	14/20*	3/19	10/14
35	15/20	10/20*	1/14	5/10
60	14/20	14/20*	0/14	6/14

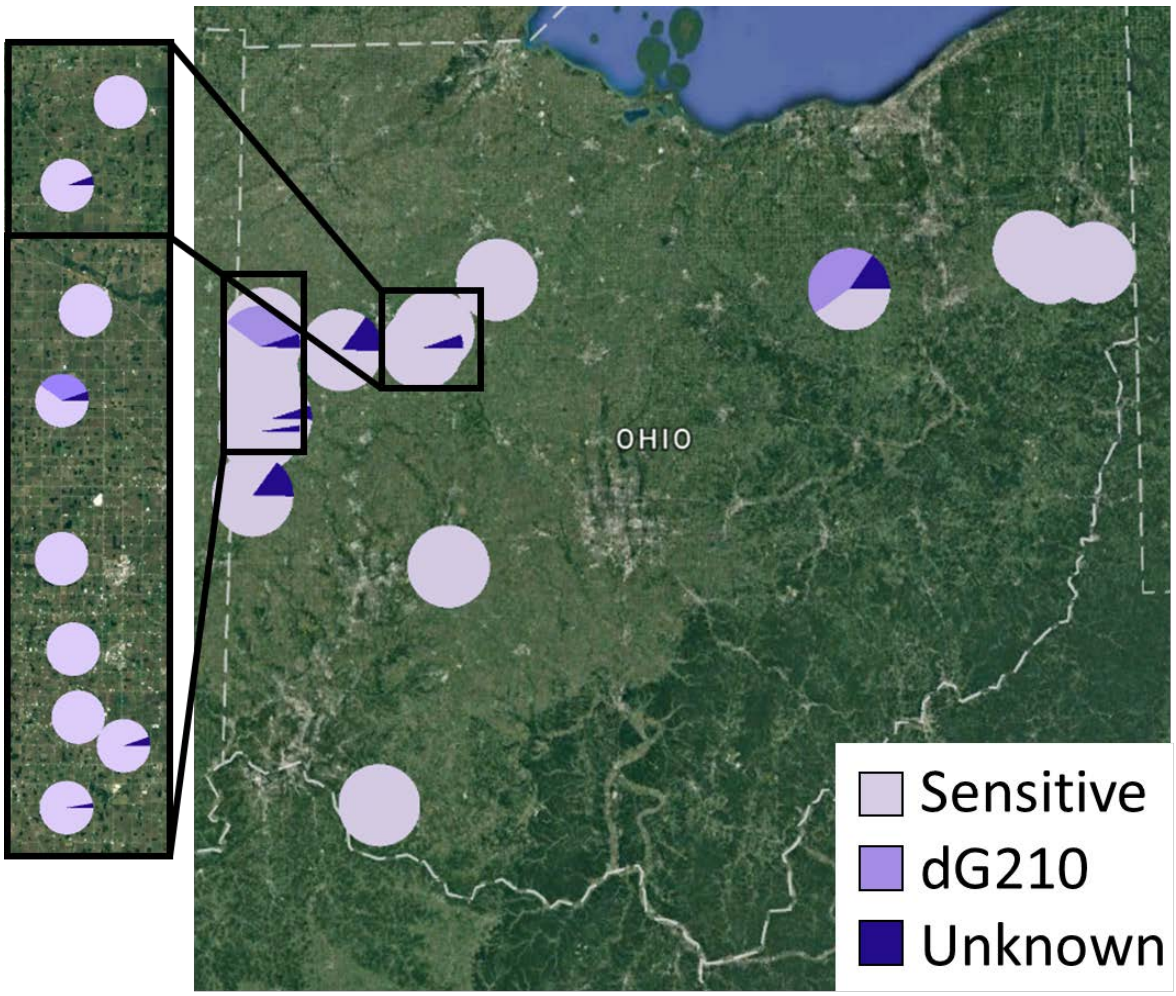

Figure 4.3. Distribution of PPO-inhibitor resistance mechanisms within *A. tuberculatus* populations collected during the Ohio state-wide survey. No R128 mutations were observed within any population.

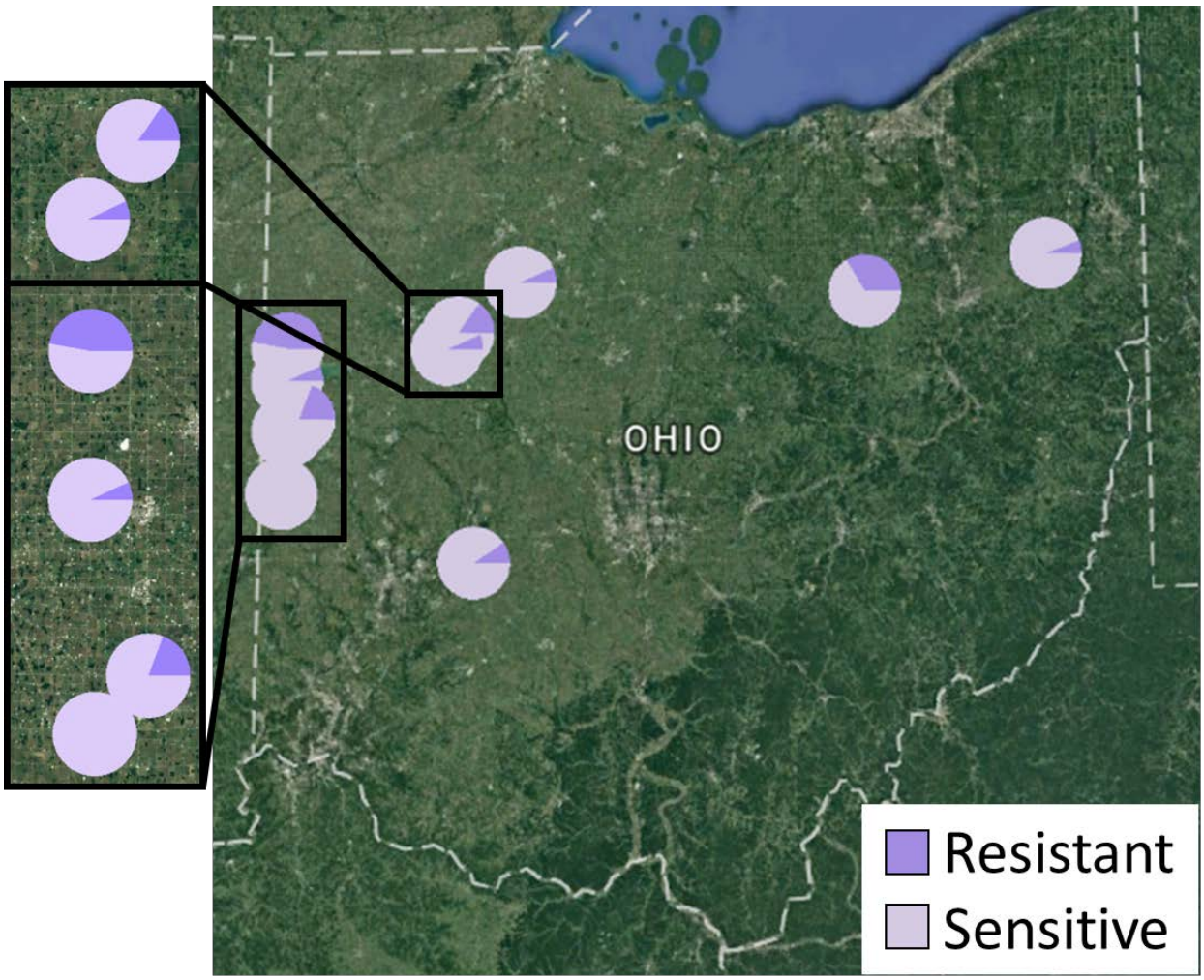

Figure 4.4. Distribution of Atrazine resistance within *A. tuberculatus* populations collected during the Ohio state-wide survey. No G264S mutations were observed within any population.

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CHAPTER 5: CHARACTERIZATION OF HERBICIDE-RESISTANT *AMARANTHUS RETROFLEXUS*

ABSTRACT

Amaranthus retroflexus is one of the most widely distributed agricultural weeds and reported to cause severe crop losses. Unlike related species, such as *Amaranthus tuberculatus*, relatively few cases of herbicide resistance have been reported for *A. retroflexus*. EPSPS-inhibitors such as glyphosate and PPO-inhibitors such as fomesafen are commonly used to control *A. retroflexus*. In this study, accessions of *A. retroflexus* from Ohio were screened for resistance to these herbicides and resistant phenotypes were characterized. A resistance factor of 2.2 was observed to PPO-inhibitor fomesafen between two *A. retroflexus* accessions. An investigation of the target site, the *PPX2* gene, revealed two amino acid substitutions that delimited resistant and sensitive phenotypes: D414N and C277S. No mutations known to cause resistance to PPO-inhibitors was observed within the resistant accession. Intriguingly, a third accession possessed a potentially intermediate phenotype between the resistant and sensitive accession and was found to be heterozygous at both positions. Resistance to EPSPS-inhibitor glyphosate could not be confirmed in this study. This is the first report of PPO-inhibitor resistant *A. retroflexus* within the USA. Continued resistance screening is necessary to identify new resistance cases and characterize new resistance mechanisms.

INTRODUCTION

Amaranthus retroflexus is a monoecious, annual weedy species of ruderal and agricultural habitats (Sauer 1967; Holm et al 1997; Costea et al 2004; Mandak e al 2011). Originating from central and eastern USA, southeastern Canada, and northeastern Mexico, *A.*

retroflexus is currently naturalized worldwide (Sauer 1967; Holm et al 1997). In an agricultural context, *A. retroflexus* is an economically important weed species throughout much of the temperate regions of the northern and southern hemispheres, including Canada, US, Europe, India, Australia, and Poland (Sauer et al 1967; Holm et al 1997). Furthermore, yield losses in soybean have been reported up to 38% as a result of *A. retroflexus*. Yield loss from *A. retroflexus* competition, as with other weedy species, is dependent on crop, emergence time relative to crop, weed density, and level of control (Knevic et al 1994; Dieleman et al 1995; Bensch et al 2003).

A variety of herbicides across many sites of action (SoA) have been reported efficient in the control of *A. retroflexus*, such as acetolactate synthase (ALS), protoporphyrinogen oxidase (PPO) (Mayo et al 1995), microtubule (Coetzer et al 2002), psbA (Naleqaja and Adamcsewski 1977), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (Sutton et al 2002). Perhaps in response to an increased reliance on herbicide control, multiple herbicide resistant populations of *A. retroflexus* have been reported worldwide (Heap 2018). Multiple target site resistance alleles have been observed in *A. retroflexus* populations (McNaughton et al 2004) with no fitness cost (Sibony and Rubin 2003). Cases of resistance have been observed for inhibitors of *psbA* (Oettmeier et al 1982). More troubling, reports of resistance to PPO-inhibitors are becoming increasingly frequent in recent years, with reports from Brazil in 2014 and China in 2017, though no mechanisms have been proposed (Heap 2018).

Inhibition of PPO leads to an accumulation and export of protoporphyrin IX from organelles to the cytoplasm. In the presence of light, protoporphyrin IX induces the formation of singlet oxygen, a reactive oxygen species which results in plant death (Patzoldt et al 2006). Resistance to PPO-inhibitor herbicides has been observed in related *Amaranthus* spp., such as

Amaranthus palmeri and *Amaranthus tuberculatus* (Heap 2018). Several mechanisms of resistance have been characterized, including a G210 deletion (Patzoldt et al 2006) and several substitutions at the R128 position of *A. palmeri* (Giacomini et al 2017). Interestingly, the G210 deletion is hypothesized to be very unlikely to occur in *A. retroflexus* due to the lack of the microsatellite site that is likely necessary for the deletion (Riggins and Tranel 2012). Amino acid variation at the R128 position within *A. retroflexus* is unknown.

The identification of new mechanisms of resistance is necessary for the development of high-throughput screening methods to avoid intensive and costly phenotyping. Additionally, the identification of new resistance mechanisms provides a greater understanding of herbicide-protein interactions, as key amino acid residues for herbicide binding are frequently identified in the case of target site resistance. Finally, the emergence of new resistance mechanisms over time has evolutionary implications. Why did certain resistance mechanisms appear 'faster' than others? Are these alleles naturally present within the population at a very low frequency, and it issimply management decisions and starting allele frequencies that are responsible for this separation, or are these mechanisms evolving in response to the herbicide selection pressure?

In this study, accessions of *A. retroflexus* collected from Ohio were screened and characterized for resistance to the herbicides fomesafen and glyphosate. Our objective was to identify herbicide-resistant accessions of *A. retroflexus* though rapid screening, and characterize resistant populations through dose-response assays and molecular investigation to identify candidate mechanisms involved in resistance.

MATERIALS AND METHODS

Seed Collection

Amaranthus retroflexus seed was collected during a late-season survey of weed infestations in soybean fields. Seed of each collected plant was initially screened for resistance to glyphosate and fomesafen in trays. Three accessions, BM3, BM4, and BM5 were selected based on initial screening.

Plant Growth Conditions

Seeds were surface sterilized with 50% fresh bleach, washed with deionized water and kept at 4ºC for five weeks before sown. Plants were germinated in Petri dishes at 35ºC with 12-hour days. Seedlings were transplanted into trays for initial screening, and into individual cone-tainers (Stuewe and Sons Inc) for dose responses. All soil used in this study was a 3:1:1:1 Sunshine LC1: soil:peat:torpedo sand blend. Plants were kept in the greenhouse at 12-hour days, 28-30ºC during the day and 25-27ºC at night. Plants were watered twice daily with mist irrigation.

Herbicide Application

Plants from each population in the 4- to 6-leaf stage were selected for uniformity. Herbicide applications were made with glyphosate (Roundup Weathermax, Monsanto Company) where a 1x field rate is considered 840 g ae ha^{-1} and fomesafen (Flexstar, Syngenta Crop Protection), where a 1x field rate is considered 328.8 g ha^{-1}. Additives were included based on label requirements. Plants were sprayed using a moving-nozzle cabinet spray chamber using a 80015 even flat fan nozzle (TeeJet Technologies). Spray volume was calibrated for 187 L ha^{-1}, applied 46 cm above the plant canopy.

Initial screening with glyphosate was conducted with the following dose response: 0.5x, 1x, and 4x field rate. Initial screening with fomesafen was conducted with the following dose response: 0.5x, 1x, and 4x field rate. Initial screening was performed twice, each dose was evaluated on at least eight plants per accession. Three weeks after herbicide application, survivorship and growth response were visually assessed. A putative strong resistance response is observed when consistent survivorship per dose is observed, and survivors are morphologically comparable to the next lowest dose. A putative moderate resistance response observes consistent survivorship per dose, but increased injury as doses increase. A putative susceptible response observes a decrease in survivorship as dose increases.

Further resistance characterization with glyphosate and fomesafen was conducted with a ten step, two-fold dilution series from 2x field rate, and an untreated control, for a total of eleven treatments. Each dose response was conducted twice, each time with at least six plants per accession per dose. Plants for all dose response experiments were organized in a completely randomized design. Three weeks after glyphosate treatment, and two weeks after fomesafen treatment, each plant was separately harvested for above-ground biomass. All biomass was dried for 7 days at 37^{o}C and weighed.

Dose Response Modelling

Separate dose response models were constructed for each herbicide. Dry biomass data relative to untreated controls for each accession was fit to a 4-parameter log-logistic model using the drc package in R (Ritz et al 2015). Variance was normalized through 4th root transformation, as recommended though the boxcox procedure. Significance of each parameter was tested using available functions within the package.

DNA Extraction

Single-leaf samples were taken from three untreated plants per accession prior to herbicide application. Hexadecyltrimethylammonium bromide (CTAB) DNA extraction procedure (Doyle and Doyle 1990) was conducted on all leaf samples. Samples were assessed for quality and DNA quantity using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). All DNA samples were diluted to 10ng μL^{-1} for molecular diagnostics.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from three untreated plants per accession using the TRIzol-based RNA extraction method (Thermo Fisher Scientific). cDNA was synthesized using Protoscript First Strand cDNA Synthesis Kit (New England BioLabs).

Sequencing of *PPX2*

In the absence of *PPX2* gene sequence of *A. retroflexus*, primers were designed off the *A. tuberculatus* cDNA sequence DQ386117 (Table 5.1). Primers encompass base pairs (bp) 83-1369 of a predicted 1605 bp from the reference sequence. PCR was conducted using GoTaq Flexi DNA Polymerase under the following conditions: 1.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.1µM each primer (Table 5.1), 1.25u polymerase, 2.5ug cDNA template. Thermocycler program was as follows: 1 cycle of 95ºC for 2 minutes, 35 cycles of: 95ºC for 30 sec, annealing for 30 sec, 72ºC for 1 min. Finally, thermocycler program was terminated with 72ºC for 5 minutes. PCR products were purified using the E.Z.N.A. cycle-pure kit (Omega Bio-tek). Purified product was used for Sanger sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

SNP Calling

Sequences were visually curated using DNA Baser chromatograph editor (Heracle BioSoft). Sequences were aligned with reference *Amaranthus hypochondriacus PPX2* open reading frame EU024569.1 (known to be sensitive to PPO-inhibitors) using MAFFT 7.311 on default settings and visualized with Jalview (Waterhouse et al 2009). SNPs that delimited between resistant and susceptible accessions and resulted in protein modifications were identified visually.

RESULTS AND DISCUSSION

Resistance ratings obtained from initial screenings are recorded in Table 5.2. Accession BM3 was rated the most sensitive to fomesafen and glyphosate. Accession BM4 was rated intermediately resistant to fomesafen and sensitive to glyphosate. Accession BM5 was putatively resistant to glyphosate. Visually distinct resistance at 0.5x field rate glyphosate application between BM5 was sufficient to label the accession as 'putatively resistant' to glyphosate (Table 5.2). Resistance was characterized between the following pairs of accessions: for glyphosate, BM4 and BM5; for fomesafen, BM3 and BM5. BM4 was subjected to a fomesafen dose response, however it was not replicated due to seed germination issues.

A fomesafen dose response of accessions BM3 and BM5 supports observations of the initial screening (Figure 5.1). Models constructed from the relative dry weight identify two significant parameters: 'ED50' and 'Lower Limit' (Table 5.3). A significant ED50 value is required to confirm resistance within an accession when compared to a sensitive control. In the case of accession BM5, the resistance factor, or fold increase in dose required to result in a 50% inhibition of growth, compared to the sensitive accession, is 2.2. While no characterized

resistance mechanism has been reported in *A. retroflexus*, several mechanisms found within the *Amaranthus* genus can be used for comparison. Salas et al (2016) reported resistance factors to fomesafen of 6, 13, and 21 in populations possessing the G210 deletion resistance mechanism. Patzoldt et al (2005) reported a resistance factor to fomesafen of 6.2 in a population possessing the ΔG210 resistance mechanism, and reported between 2.2 and 23-fold resistances to assorted PPO-inhibiting herbicides. While the observed 2.2-fold resistance in *A. retroflexus* may be on the low end of this range, this level of resistance was sufficient for survival at 1x field rate in trays (Table 5.2). The initial screening suggests that 2.2-fold resistance may be sufficient for a loss of control of this accession under field conditions. A significantly increased lower limit does not typically have major implications for in-field resistance, however it may provide insight into the mechanism involved in resistance. An increased lower limit can be considered the result of additional above-ground biomass accumulation prior to plant death. Multiple possibilities exist which can explain this phenomenon. The plant, in response to the chemical, could increase the rate of biomass production per unit time, sending more carbon to the above ground tissue. Alternatively, plant death is delayed, potentially indicating a slowed movement of the herbicide to its site of action. A resistant plant may have additional mechanisms to deal with the results of target site inhibition, such as increased radical scavenging capacity, resulting in a delay in plant death. The increase in biomass may be completely distinct from a resistance mechanism, where characteristics of the accessions result in differing growth rates.

Here, we report the first partial *PPX2* gene sequence of *A. retroflexus* (Table 5.4). Sequence analysis of the *PPX2* gene of *A. retroflexus* accessions revealed two amino acid substitutions which delimited R and S phenotypes: D414N and C277S (Table 5.5) when aligned to *A. hypochondriacus*. Intriguingly, the accession BM4, which displayed an intermediate

phenotype in both initial screenings and in an un-replicated characterization dose response, was heterozygous at both positions. No known mutations, including at position R128 (Giacomini et al 2017) were observed. The D414N substitution, resulting from a G1237A mutation, is rarely observed within other plant species. In fact, across a panel of 98 chloroplastically targeted *PPX* genes, only sequences originating from cocoa and two species of cotton possess the 413N amino acid (Dr. Franck Dayan, personal communication). While the response of cocoa to fomesafen is unknown, cotton is a labelled crop for fomesafen application. The mechanism of tolerance to fomesafen in cotton is unknown. In the case of C227S, the locus is poorly conserved through the available diversity of the *PPX* genes, preventing comparison across a wide species range. In the case of both mutations, functional complementation in a model system, such as in *E. coli*, is necessary to determine if either or both mutations are sufficient to produce the resistant phenotype observed biologically (Patzoldt et al 2006).

A glyphosate dose response of accessions BM4 and BM5 produced a similar response to increasing rates (Figure 5.2). Furthermore, no significant difference in parameter estimates were observed (Table 5.6), indicating the 'putative' resistance observed during the initial screening may be a result of visual evaluation instead of a quantitative procedure.

In the case of PPO-inhibitor resistant *A. retroflexus*, the lack of known resistance mechanisms within the *PPX2* gene indicates that a new mechanism of resistance is responsible for the phenotype observed in the dose response experiments. While untested amino acid substitutions exist within the target site of PPO-inhibitors, alternative mechanisms could be at work. Further follow-up is required to fully elucidate the resistance mechanism.

The accessions investigated in this research were collected as part of a state-wide survey of Ohio. This survey specifically targeted soybean fields infested with *Amaranthus* spp.

Continued surveillance is necessary to identify resistant populations prior to allow management, and potentially eradication, efforts to be conducted.

In conclusion, a PPO-inhibitor resistant accession of *A. retroflexus* has been identified and characterized. The resistant accession is 2.2 fold more resistant to fomesafen than the sensitive control. The first partial sequence of the *PPX2* gene of *A. retroflexus* is reported (Table 5.4). Sequence analysis identified two potential target-site SNP which result in amino acid modifications delimiting resistant and susceptible accessions. To the best of our knowledge, this is the first report of PPO-inhibitor resistant *A. retroflexus* within Ohio, and the US as a whole. Continued screening for resistant populations is necessary for the identification of new resistance mechanisms within weedy populations.

TABLES AND FIGURES

Table 5.1. Primer design for *PPX2* cDNA of *A. retroflexus*.

Name	Sequence	Annealing Temperature
RR-PPOF1	TAGCTGTAATGGGCAACATTTC	53.5C
RR-PPOR1	CCAAGATCATCGATCAAATCTG	
RR-PPOF4	CTAGAGCTGGAGGCAAACTTA	54.3C
RR-PPOR4	CATAACGGAAAGGGGTACGT	
RR-PPOF6	GGTACATGTGGTGGAGATCCT	55.6C
RR-PPOR6	AAGCCCTCAAGAGGTCTCT	
RR-PPOF7	CTGTGGTTGTCACTGCTCCAA	57.4C
RR-PPOR7	ATGAAGGTTCGTCCTCAGTGC	

Table 5.2. Resistance ratings[a] for *A. retroflexus* accessions from initial screening

	Fomesafen (field rate)			Glyphosate (field rate)		
Accession	0.5x	1x	4x	0.5x	1x	4x
BM3	R	S	S	r	S	S
BM4	R	r	S	r	S	S
BM5	R	R	S	R	S	S

[a] Resistance rating scales (most sensitive to most resistant): S, r, R

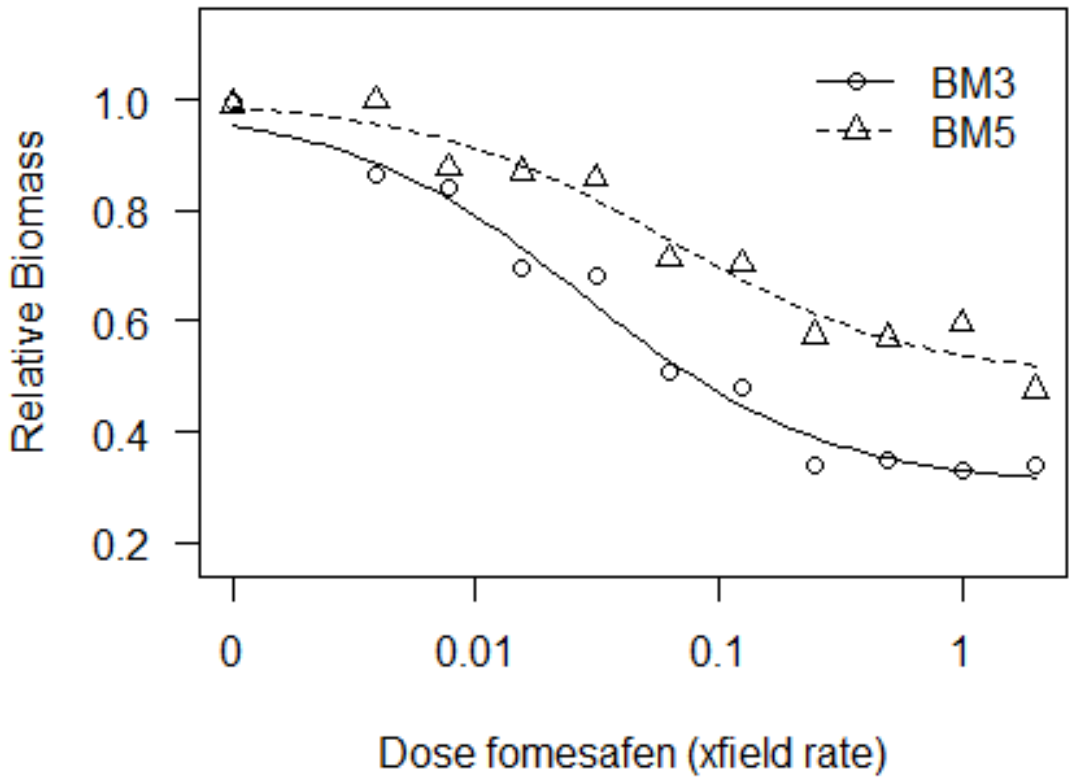

Figure 5.1. Dose response of resistant and sensitive *A. retroflexus* accessions to fomesafen. Doses are reported based on field rate (1x = 328.8 g ha^{-1}). Biomass is relative to untreated control (0x field rate), 4^{th} root transformation was conducted to correct for normality.

Table 5.3. Parameter estimates and comparisons for fomesafen dose response models.

Population	Parameter	Estimate	Std. Error	Comparison (P-value)
BM3	Upper Limit	0.987	0.0366	0.809
BM5		0.999	0.0337	
BM3	Lower Limit	0.303	0.0327	0.0000314[a]
BM5		0.495	0.0512	
BM3	ED50	0.0279	0.00633	0.00508[a]
BM5		0.0622	0.0230	
BM3	Slope at ED50	0.883	0.153	0.915
BM5		0.853	0.231	

[a] significant at P-value < 0.05

Table 5.4. *Amranthus retroflexus* partial cDNA consensus sequence (1410 bp) derived from a PPO-inhibitor sensitive accession.

CTGTTGTTGGTGCTGGAGTTAGTGGACTTGCTGCAGCATATAAGCTAAAATCCCATGGTTTGAA
TGTGACATTGTTTGAAGCTGATTCTAGAGCTGGAGGCAAACTTAAAACTGTTAAAAAAGATGGT
TTTATTTGGGATGAGGGGGCAAATACTATGACAGAAAGTGAGGCAGAGGTCTCGAGTTTGATCG
ATGATCTTGGACTTCGTGAGAAGCAACAGTTGCCAATTTCACAAAATAAAAGGTACATAGCTAG
AGATGGTCTTCCGGTGCTACTACCTTCAAATCCCGCTGCACTGCTCTCGAGCAATATCCTTTCA
GCAAAATCAAAGCTGCAAATTATGTTGGAACCATTTCTCTGGAGAAAACGCAATGCTACTGAGC
TTTCTGATGAGCATGTTCAGGAAAGCGTTGGTGAATTTTTTGAGCGACATTTTGGGAAAGAGTT
TGTTGATTATGTTATTGACCCTTTTGTTGCGGGTACATGTGGCGGAGATCCTCAATCGCTATCT
ATGCACCATACATTTCCCGACGTATGGAATGTTGAAAAAAGGTTTGGCTCTGTGTTTGCTGGAC
TAATTCAATCAACATTGTTATCTAAGAAGGAAAAGGGTGGAGGAGAAAATGCTTCTATCAAGAA
GCCTCGTGTACGTGGTTCATTTTCATTCCATGGTGGAATGCAGACACTTGTTGACACAATGTGC
AAACAGCTTGGTGAAGATGAACTCAAACTCCAGTGTGAGGTGCTGTCCTTGTCATACAACCAGA
AGGGGATCCCTTCATTAGGGAATTGGTCAGTCTCTTCTATGTCAAATAATACCAGTGAAGATCA
ATCTTATGATGCTGTGGTTGTCACTGCTCCAATTCGCAATGTCAAAGAAATGAAGATTATGAAA
TTTGGAAATCCATTTTCACTTGACTTTATTCCAGAGGTGACTTACGTACCCCTTTCCGTTATGA
TTACTGCATTCAAGAAGGATAAAGTGAAGAGACCACTCGAGGGCTTCGGAGTTCTTATCCCGTC
TAAAGAGCAACATAATGGACTGAAGACTCTTGGTACTTTATTTTCCTCTATGATGTTTCCCGAT
CGTGCTCCATCTGACATGTGTCTCTTTACTACATTTGTCGGAGGAAGCAGAAATAGAAAACTTG
CAAAAGCTTCAACGGATGAATTGAAGCAAATAGTTTCTTCTGACCTTCAGCAGCTGTTGGGCAC
TGAGGACGAACCTCTTCATGTCAATCATCTCTTTTGGAGCAATGCATTCCCGTTGTATGGGCAC
AATTACGATTCTGTTTTGAGAGCAATAGACAAGATGGAAAAAGATCTTCCCGGATTTTTTTATG
CAGGTAACCATAAGGGCGGACTTTCAGTCGGAAAAGCGATGGCCTCCGGATGCAAGGCTGCAGA
AC

Table 5.5. Amino acid substitutions delimiting PPO-inhibitor resistance in *A. retroflexus* based on *A. hypochondriacus* open reading frame.

Position (amino acid)	BM3 (S)	BM4 (R)	BM5 (R)
277	C	S	S
414	D	N	N

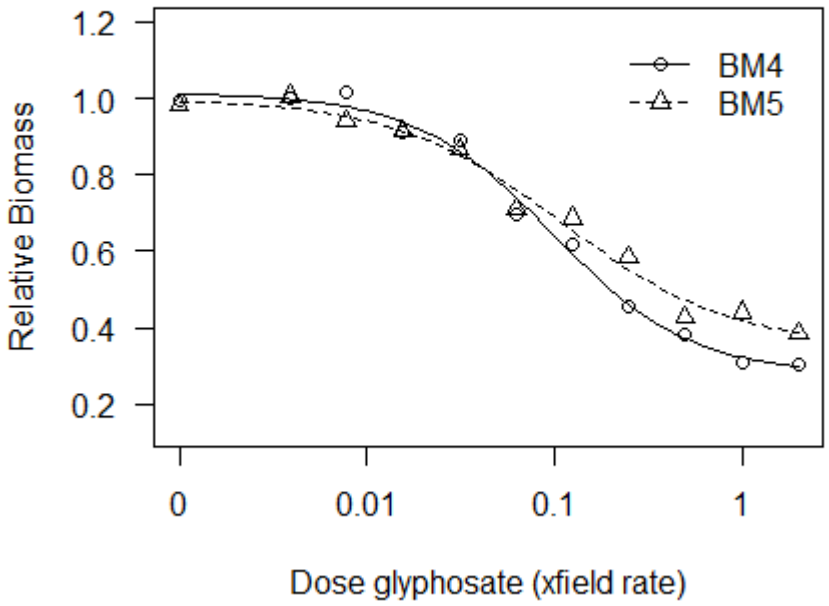

Figure 5.2. Dose response of *A. retroflexus* accessions BM4 and BM5 to glyphosate. Doses are reported based on field rate (1x = 840 g ai ha^{-1}). Biomass is relative to untreated control (0x field rate), 4^{th} root transformation was conducted to correct for normality.

Table 5.6. Parameter estimates and comparisons for glyphosate dose response models.

Population	Parameter	Estimate	Std. Error	Comparison (P-value)
BM4	Upper Limit	1.01	0.0311	0.751
BM5		0.999	0.0355	
BM4	Lower Limit	0.278	0.0483	0.302
BM5		0.350	0.0633	
BM4	ED50	0.0971	0.0201	0.728
BM5		0.110	0.0349	
BM4	Slope at ED50	1.18	0.240	0.580
BM5		0.970	0.235	

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CHAPTER 6: ARTIFICIAL HYBRIDIZATION BETWEEN *AMARANTHUS TUBERCULATUS* AND *AMARANTHUS ALBUS*

ABSTRACT

The *Amaranthus* genus includes highly successful agricultural weeds originating in North America. Each species within the genus possesses a unique geographical range in addition to key traits. The majority of herbicide resistance traits present within the genus are concentrated on few key species: *Amaranthus tuberculatus* and *A. palmeri*. Species with overlapping geographical ranges possess favorable weedy characteristics not present in *A. tuberculatus* or *A. palmeri*, such as the tumbling seed dispersal mechanism of *A. albus*. The hybridization potential between *A. tuberculatus* and *A. albus* was determined in a controlled environment. From 64,758 seeds effectively screened from *A. albus* grown in proximity to *A. tuberculatus* males, a total of three hybrids could be confirmed through screening for ALS-inhibitor resistance trait and molecular markers: a hybridization rate of 0.0046%. The hybrids possessed morphology intermediate between both parents, however dioecy and 'erectness' appeared dominant. A female putative hybrid was backcrossed to *A. tuberculatus* and progeny were evaluated for DNA content. Due to elevated DNA content in the BC1 population compared to either parent, polyploidization may be required for hybridization. Screening of seeds from *A. tuberculatus* females grown in proximity with *A. albus* recovered no hybrids; however, a significant female gender bias was observed. The potential unidirectional gene flow from *A. tuberculatus* to *A. albus* observed in this study highlights a new avenue for the gain of herbicide resistance by *A. albus*.

INTRODUCTION

The *Amaranthus* genus contains some of the most successful agricultural weeds to have originated from North America (Sauer 1967; Trucco and Tranel 2011; Heap 2014). Perhaps most notable is the rapid accumulation of herbicide resistance traits within two key members of the genus: *Amaranthus tuberculatus* and *A. palmeri* (Heap 2014; Heap 2018). In fact, a considerable diversity of traits exists within the genus. *A. retroflexus* has successfully naturalized world-wide, flourishing under a wide variety of environments (Sauer 1967). *Amaranthus palmeri* has a notably high photosynthetic capacity which exceeds 70 μmol CO_2 m^{-2} s^{-1} (Ehleringer 1983). *A. albus* is a tumbleweed, allowing for long distance seed dispersal, whereas most other *Amaranthus* spp are erect (Costea and Tardif 2003). In addition to unique traits, each species possesses a unique geographical range. For instance, *A. palmeri* is not native to the northern United States, while *A. retroflexus* is native to the entire USA (PLANTS-USDA 2018).

Within the overlapping regions of the geographic range of two species, the potential for hybridization exists. A review of hybridization potential within the genus *Amaranthus* is provided by Trucco and Tranel (2011). In summary, hybridization has been observed between an assortment of species within the genus, under both controlled environments and in field conditions. Unidirectional gene flow from *A. tuberculatus* to *A. hybridus* was observed to transfer resistance to acetolactate synthase (ALS)-inhibiting herbicides in field conditions (Trucco et al 2005). *Amaranthus palmeri* was observed to transfer resistance to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-inhibiting herbicides to *A. spinosus* under field conditions (Nandula et al 2014). *Amaranthus palmeri* was observed to transfer resistance to ALS-inhibitors to *A. tuberculatus* at low frequencies under a controlled environment (Franssen et al 2001). Herbicide resistance traits are often a target for these hybridization studies as progeny

screening is straightforward (herbicide selection discriminates putative hybrids) and provides practical implications.

The transfer of herbicide resistance traits between species influences the spread of herbicide resistance. Hybridization between two species with distinct but overlapping geographic ranges can result in the movement of herbicide resistances outside of a predictable area. Furthermore, should herbicide resistance transfer from a 'resistance reservoir' such as *A. tuberculatus*, to a species with the potential for wide-range seed dispersal, such as the tumbleweed *A. albus*, any transferred resistance genes could become widespread in a relatively short period of time. In the case of another tumbleweed, *Kochia scoparia*, resistance to *EPSPS*-inhibitors was first introduced to Southern Alberta in 2011, but by 2013 it was observed across the entire Canadian prairie (Beckie et al 2015).

The purpose of this study was to determine the hybridization potential of *A. albus* and *A. tuberculatus*. Due to the great difference between the phenotypes of the selected species, the dominance of distinguishing morphological traits, such as erect vs tumbleweed, were determined. The potential for gene introgression was investigated, and requirements for hybridization to occur were investigated.

MATERIALS AND METHODS

Population Generations

An ALS-inhibitor resistant *A. tuberculatus* population and an ALS-inhibitor sensitive *A. albus* population were selected. The ALS-inhibitor resistance was due to a single amino acid substitution, W574L. The populations were allowed to cross reciprocally under pollen containment tents in greenhouses. To further limit contamination, all crosses were conducted in

the winter to limit outside pollen presence. Seed was collected through manual threshing. Seeds were surface sterilized with 50% fresh bleach, washed with deionized water and kept at 4ºC for five weeks in a 0.1% agarose solution. Hybrid plants were backcrossed to the *A. tuberculatus* parent under open pollinated conditions, producing the BC1 population.

Hybrid Screening

Seeds from *A. albus* plants pollinated with *A. tuberculatus* were screened for resistance to the ALS-inhibiting herbicide imazethapyr (Pursuit, BASF). Seeds were seeded at 39 seeds cm^{-2} in flats. Total seeds planted was determined by seed weight. Percent germination was calculated based on germination counts conducted in petri dishes to determine number of seed screened. All seeds from *A. albus* were screened with 360g ai ha^{-1} PRE application, the equivalent of a 4x field rate. Flats were sprayed using a moving-nozzle cabinet spray chamber using a 80015 even flat fan nozzle (TeeJet Technologies). Spray volume was calibrated for 187 L ha^{-1}, applied 46 cm above the soil surface. Survivors were classified as putative hybrids and screened with molecular markers (Wetzel et al 1999). Putative hybrids were morphologically characterized. As *A. tuberculatus* is dioecious, all seed produced from the *A. tuberculatus* pollinated with *A. albus* was treated as putative hybrids and screened with molecular markers (Wetzel et al 1999).

Marker Analysis

Restriction fragment length polymorphism (RFLP) markers specific to *A. albus* and *A. tuberculatus* were used as described by Wetzel et al (1999) on all putative hybrids. This assay consists of restriction enzyme digests of the internal transcribed spacer (*ITS*) region. Expected digest patterns for each species and hybrid are described in Table 6.1.

DNA Content Analysis

Putative hybrids were backcrossed to the *A. tuberculatus* parent and seed harvested. Plants were grown to flowering, and branches of three progeny were harvested for DNA content analysis. Nuclei isolation and flow cytometry were conducted as described by Rayburn et al (2005) with the following modifications: maize hybrid VT3 was used as an internal standard for each sample. Peak area was calculated using FCS Express software (De Novo Software).

RESULTS AND DISCUSSION

Eighty-six thousand seeds by weight sourced from eight *A. albus* plants were screened with imazethapyr. A germination rate of 75.3% was calculated, resulting in an estimated 64,758 seeds effectively screened. A total of 13 survivors were obtained, and were screened with molecular markers (Wetzel et al 1999). Ten of the 13 survivors were determined to be *A. tuberculatus* contamination. The remaining three plants successfully passed the molecular marker screening (Figure 6.1), confirming their status as hybrids. A unidirectional hybridization rate of 0.0046% was observed from *A. tuberculatus* to *A. albus*. In comparison to previous literature, *A. tuberculatus* to *A. hybridus* crosses resulted in approximately 5% hybridization rate under field conditions (Trucco et al 2005), while *A. palmeri* to *A. tuberculatus* crosses resulted in a hybridization rate of 1% (Franssen et al 2001). Interestingly, a phylogenetic analysis of the *Amaranthus* genus placed *A. albus* as being more closely related to *A. tuberculatus* than *A. tuberculatus* is to *A. palmeri* (Stetter and Schmid 2017). The 2N chromosome number of *A. albus* is 32, as is *A. tuberculatus* (Grant 1959). *A. tuberculatus* appears to have a higher rate of hybridization with *A. palmeri* (2n = 34) than with *A. albus* (Grant 1959; Franssen et al 2001). Several reasons could explain the low frequency of hybridization observed in this study. *A. albus*

pollen may be very competitive and fast growing on a suitable host. Alternatively, pre-zygotic barriers may exist severely inhibiting the ability of *A. tuberculatus* pollen from fertilizing *A. albus* eggs. A detailed understanding of pollen tube growth differences within the *Amaranthus* genus, including their hybrids, is lacking from the literature.

The morphology of the mature hybrids are shown in Figure 6.2. A varying degree of branching was observed in all cases. In the case of hybrids HY2F and HY3M, branching was primarily observed once the plant became reproductive. HY1S began branching during vegetative stages. While more branching was observed in putative hybrids than typical for *A. tuberculatus* under the growing conditions, all putative hybrids exhibited a clear erect growth pattern distinct from the *A. albus* parent. This suggests that the erect phenotype is dominant to tumble. Continued backcrossing to a 'tumble' background would be required to obtain both herbicide resistance in addition to a tumbleweed phenotype. In terms of fertility, HY1S was a fully sterile female while HY2F was a fertile female. HY3M was male. In all cases, putative hybrids were dioecious. The dominance of dioecy has considerable support from the available *Amaranthus* literature, specifically from crosses between *A. tuberculatus* and other amaranths (Trucco et al 2005). Stem color, which was distinct between parents, was split amongst the obtained putative hybrids. HY1S and HY3M had white-green stems, similar to the *A. albus* parent, while HY2F had a red stem, similar to the *A. tuberculatus* parent. Leaf shape was consistent with the *A. tuberculatus* parent. In the case of all hybrids, leaves were observed throughout the inflorescence, a characteristic of *A. albus*. The presence of morphological traits from both parents provides substantial evidence that these plants are true hybrids. Additionally, morphological characteristics from both parents indicates that both genomes are transcriptionally

active. Given an appropriate selection pressure, selection for hybrid progeny could occur in the wild.

Molecular screening of 120 seed from four *A. tuberculatus* females resulted in no hybrids being observed. Intriguingly, a large female bias was observed in the gender ratios of the progeny (Table 6.2). The gender ratios observed deviated significantly from the expected 1:1 male:female ratio, as demonstrated by a Chi-squared test conducted within each population (Table 6.2). Approximately 87% of viable progeny were female. Two explanations have been previously proposed for such a phenomenon. *A. tuberculatus* has the potential to 'revert' and produce male flowers on a genetically female plant (Figure 6.3), resulting in auto-pollination. Alternatively, the potential for apomixis has been proposed, where seed are produced in the absence of fertilization (Ribeiro et al 2013). Dioecy can be viewed as a limiting factor for the colonization of a new region. If only a male or a female is present within a new region, reproduction is prevented. For annual species, this represents a failed colonization event. There is a clear advantage for a dioecious species, specifically colonizers of ruderal environments, to possess mechanisms to produce progeny in the absence of an outside pollen source. In terms of the proposed mechanisms, auto-pollination provides the opportunity to recombine, similar to a selfed plant. Any heterozygous loci will segregate, resulting in genetic diversity within the next generation. In the case of apomixis, no genetic diversity is present between populations, which may be disadvantageous.

Similar to F1 parents, the BC1 populations derived from HY2F possessed morphological characteristics of both parents, as shown in Figure 6.4. A higher degree of branching was observed than what is typical of *A. tuberculatus* under the growth conditions. Leaves were observed throughout the inflorescence. These observations provide further evidence *A. albus*

genetics are present within these plants and are being expressed. Interestingly, all plants appear sterile, or possess very limited seed set (10's of seed per female plant). DNA content analysis revealed that all tested hybrid progeny possessed DNA contents greater than the literature values (Rayburn et al 2005) of either parent (Table 6.3). Increases in DNA content is indicative of a polyploidy event. Interestingly, prediction of DNA content from parental values is unable to produce a similar DNA content (Figure 6.5). Even when polyploidization occurs in only one parent, similar DNA contents cannot be obtained. As putative hybrid HY2F is fully fertile, a stable and even ploidy can be expected, such as a '4N' state. Upon backcrossing to the 2N *A. tuberculatus* parent, the resultant progeny is expected to be triploid. This triploid would be expected to be nearly, if not completely, sterile. While a triploid BC1 population is predicted to have a higher DNA content than what was observed, genetic recombination between both genomes may result in the loss of portions from either or both genomes.

Mendelian inheritance of traits possessing fitness advantages, such as herbicide resistance traits in the presence of a compatible herbicide, is not required for trait stability within a population. In the case of the gene amplification mechanism which mediates resistance to glyphosate, non-Mendelian inheritance is observed in *Kochia scoparia* (Wiersma et al 2015). However, in the course of two years, the trait went from Southern Alberta to the entire Canadian prairies (Beckie et al 2015). Furthermore, in the case of high copy number *A. tuberculatus*, some copies appear to be extrachromosomal within the nucleus (Koo et al 2018). At least in the case of herbicide resistance, presence and expression of the trait appears sufficient to allow maintenance, and potentially enrichment, across generations regardless of inheritance mechanism when under a sufficient selection pressure.

In conclusion, unidirectional gene flow was observed from *A. tuberculatus* to *A. albus* at a rate of 0.0046% under controlled environment testing. All obtained progeny were dioecious, though both fertile and sterile hybrids were obtained. While no gene flow was observed from *A. albus* to *A. tuberculatus*, the resultant progeny possessed a heavily female biased gender ratios. The dioecious *A. tuberculatus* females may be able to produce progeny in the absence of a compatible pollen source. This study demonstrates that the transfer of herbicide resistance from *A. tuberculatus* to *A. albus* is possible, though may require polyploidization events to occur. This research may assist in the characterization of new herbicide resistance cases observed in *A. albus*. Furthermore, this study adds insight into the colonization potential of *A. tuberculatus*, where simply the presence of a single female plant within a new region may be sufficient for successful colonization.

TABLES AND FIGURES

Table 6.1. Predicted restriction digest pattern of *A. albus*, *A. tuberculatus*, and their hybrids.

Species	DdeI	XhoI
A. albus	No digest	Complete digest
A. tuberculatus	Complete digest	No digest
A. albus x *A. tuberculatus* hybrid	Partial digest	Partial digest

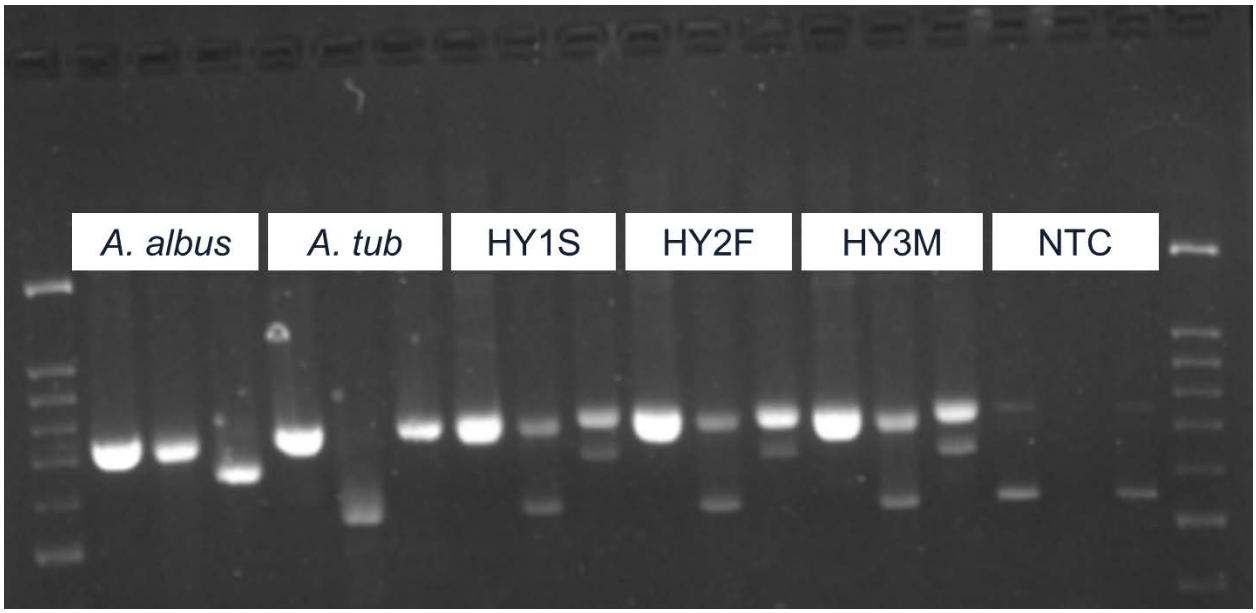

Figure 6.1. Restriction digest pattern of suspected hybrids. Band order: undigested, DdeI, XhoI.

Figure 6.2. Morphology of putative *A. albus* x *A. tuberculatus* hybrids. HY1S is sterile, and produced no seed. HY2F appeared as a fully fertile female, while HY3M appeared as a male.

Table 6.2. Gender ratios of *A. tuberculatus* female x *A. albus* progeny

Population	Male	Female	P-value [a]
ACR10	1	5	0.102
ACR20	9	36	<0.001
ACR3-5	2	21	<0.001
ACR 3-1	4	42	<0.001
Control	29	28	0.894

[a] Chi-squared test was conducted against an expected 1:1 male:female ratio.

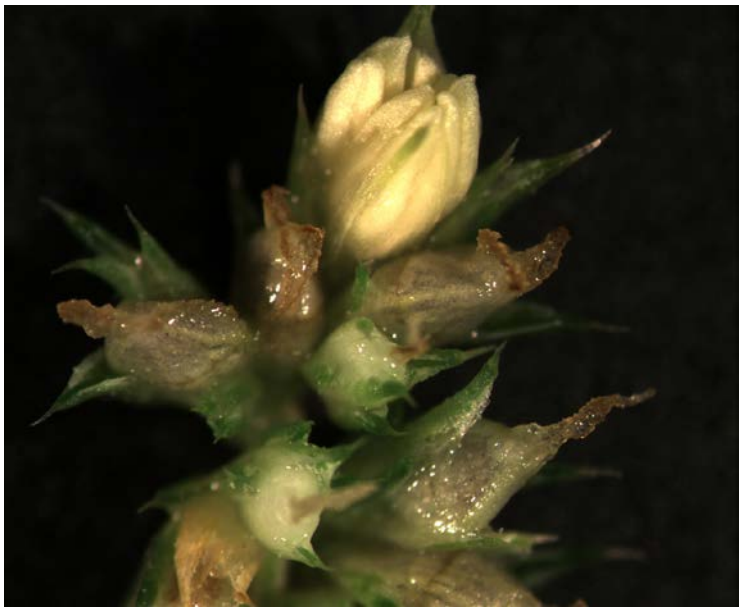

Figure 6.3. A male flower observed on an otherwise female *A. tuberculatus* plant. No female *A. tuberculatus* plants within this study were investigated for presence of male flowers. Photo was taken by Federico Trucco.

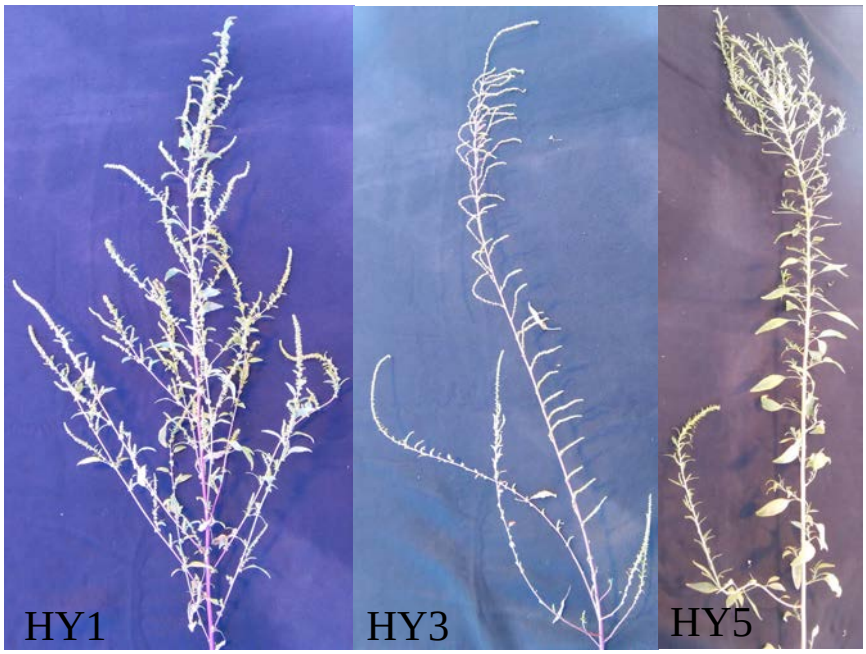

Figure 6.4. Morphology of the progeny of HY2F backcrossed to *A. tuberculatus* parent population.

Table 6.3. DNA content of BC1 population derived from putative hybrid HY2F.

Sample	Fitted Mean (sample)	fitted mean (maize)	pg DNA $2N^{-1}$
A. albus			1.18
A. tuberculatus			1.42
HY1	52199	156616	1.78
HY3	64349	185262	1.85
HY5	69967	215237	1.73

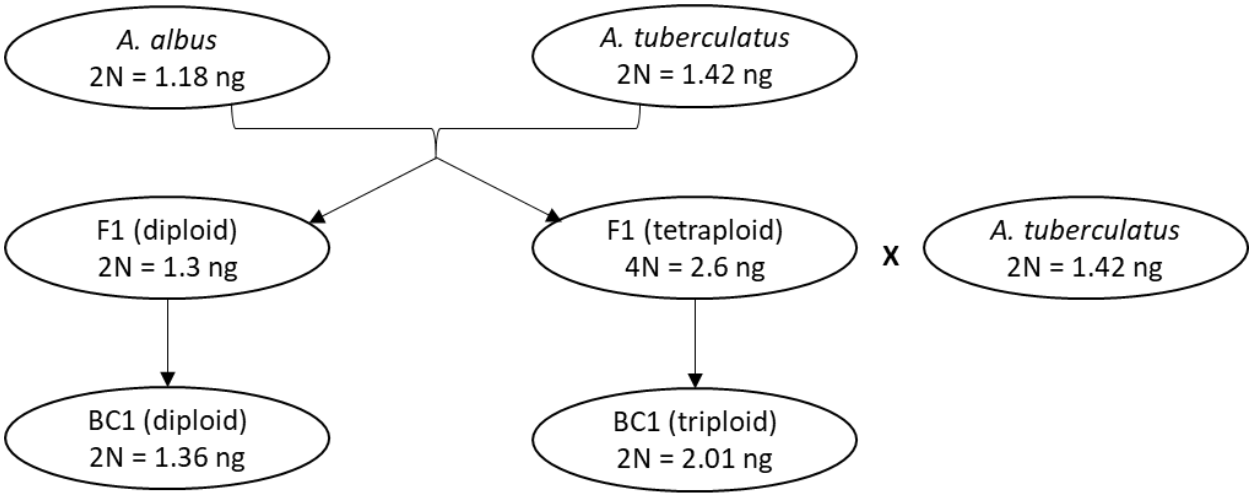

Figure 6.5. Predicted DNA content from F1 hybrid ploidy of the BC1 population, assuming proper chromosome stability and pairing.

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CHAPTER 7: CONCLUSION

Managing the spread of herbicide resistance requires understanding and quantifying key parameters, including: seed movement, gene flow, and current distribution and frequency of resistance. In this thesis, these parameters were investigated for the key driver weeds in the genus *Amaranthus*. Distribution and mechanism frequency of *Amaranthus tuberculatus* within Ohio was investigated in a state-wide survey. Gene flow between *A. tuberculatus* and *A. albus* was quantified. New, high-throughput tools to screen seedlots for *A. tuberculatus*, *A. palmeri*, and other *Amaranthus* spp. were developed. A new resistance mechanism to PPO-inhibiting herbicides was observed in *retroflexus*.

The work presented in this thesis demonstrates the importance of surveillance initiatives, utilizes their findings, and develops assays to support them. Without routine surveillance, new mechanisms of resistance as found in *A. retroflexus* may not be observed until locally problematic. Furthermore, movement of existing mechanisms into a new region may be caught early through routine surveillance. Mapping of the spread of resistance over time can not only provide insights into how these traits spread throughout a region, but also identify key factors influencing the spread. Seedlot contamination in out-of-state Conservation Reserve Program was identified through equivalent analysis. Once these factors are identified, protocols to decrease the factor's impact can be taken.

In order to identify key factors in the evolution and spread of herbicide resistance, a firm understanding of the biology and genetics of the target organism must be understood. What alleles are sufficient to produce a resistant phenotype? Are resistance mechanisms compatible with other species? Can resistance traits stably transfer between species? Can molecular assays be developed to reliably distinguish a species? Understanding these questions, which may fall

into the realm of ‘basic science’, is required to inform decision making for answers to practical questions, such as how do we mitigate the spread of herbicide resistance?

APPENDIX A: FULL PLANT REGENERATION OF *AMARANTHUS TUBERCULATUS*

INTRODUCTION

Plant transformation is a necessary procedure to answer many basic science questions, such as gene function. A plant transformation procedure can be divided into two parts, the transformation procedure and full plant regeneration in tissue culture. In the case of *Arabidopsis thaliana*, a floral dip method can be conducted, replacing tissue culture (Clough and Bent 1998). In the case of the genus *Amaranthus*, full plant regeneration procedures have been developed for a variety of agronomical, horticultural, and potentially nutraceutical accessions (Bagga et al 1987; Bennici et al 1992; Bennici et al 1997; Jofre-Garfias et al 1997; van Le et al 1998; Swain et al 2010; Pal et al 2013). Fewer accessions possess functional transformation procedures (Swain et al 2010; Pal et al 2013). However, neither plant regeneration or transformation procedures for key dioecious species *Amaranthus tuberculatus* and *A. palmeri* have been developed. To understand the underlying genetic causes of dioecy within these species, a plant regeneration and transformation procedures must be developed. In this section, the current progress on a plant regeneration procedure for *A. tuberculatus* is documented.

MATERIALS AND METHODS

Leaf Sterilization

Mature leaf tissue was collected from *A. tuberculatus* accession *WUS*. Tissue was surface sterilized in 70% ethanol for 1.5 minutes, and rinsed twice in sterile, distilled water. Tissue was subsequently submerged in 10% fresh bleach solution for four minutes, and rinsed four times in sterile distilled water, resulting in aseptic tissue.

Callus Generation

Subsamples of aseptic leaf tissue were taken, avoiding the midrib. Murashige and Skoog (MS) media supplemented with sucrose was used as a base for all tested conditions. 8.5 grams L^{-1} plant agar was used as a solidifying agent in all tested conditions. Six concentrations of the cytokinin *trans*-zeatin riboside (0, 0.35, 0.7, 1.4, 2.8, and 4.2 mg L^{-1}) and two concentrations of the auxin indole-3-acetic acid (IAA) (0, 0.9 mg L^{-1}) were tested in a factorial design for callus generation potential. Callus generation potential was assessed visually after four weeks.

Organogenesis

Callus was screened against six concentrations of *trans*-zeatin riboside (0, 0.35, 0.7, 1.4, 2.8, and 4.2 mg L^{-1}) in the absence of auxin to identify optimal organogenesis conditions. Additionally, a 26-step linear scaling of *trans*-zeatin riboside was conducted from 0 to 6.25 mg L^{-1} in the presence of 0.18 mg L^{-1} IAA. Organogenesis potential was assessed visually after four weeks.

RESULTS AND DISCUSSION

Optimal callus production was observed at 2.8mg L^{-1} *trans*-zeatin riboside and 0.9mg/L IAA (Figure A.1). Callus induction is a key step in many tissue culture protocols. Callus can be sub-divided and cultured independently to increase the quantity of materials. Once a transformation procedure has been developed, the sub-division of callus can be used to effectively clone transformation events, increasing T0 plant count.

In this study, shoot organogenesis was not observed in any tested condition. Optimal root organogenesis was observed at 4.2mg L^{-1} *trans*-zeatin riboside in the absence of IAA (Figure A.2). Continued increases in *trans*-zeatin riboside past 6 mg L^{-1} resulted in phytoxicity concerns.

To successfully obtain progeny of regenerated plants, a robust shoot and root organogenesis procedure must be produced. Typically, increases in the cytokinin-auxin ratio are necessary for shoot organogenesis to be observed. However, as *trans*-zeatin riboside was increased, phytoxicity was observed before shoot organogenesis. Continued screening with alternative, potentially less toxic cytokinin sources may be required to observe shoot organogenesis within *A. tuberculatus*.

The compatibility of *A. tuberculatus* with floral dip transformation methods has not been assessed. *A. tuberculatus* is indeterminate, allowing multiple developmental stages to be screened simultaneously. The development of a floral dip method for *Agrobacterium*-mediated transformation would result in the development of an accession compatible tissue culture method unnecessary.

FIGURES

Figure A.1. Callus induction from *A. tuberculatus* leaf segment at 2.8 mg L^{-1} *trans*-zeatin riboside and 0.9 mg L^{-1} IAA.

Figure A.2. Root organogenesis from *A. tuberculatus* callus at 4.2 mg L^{-1} *trans*-zeatin riboside.

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